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TESTOSTERONE AND ESTRADIOL-17 BETA PRODUCTION BY GOLDFISH, *CARASSIUS AURATUS*, OVARIAN FOLLICLES *IN VITRO*: INFLUENCE OF SEASON AND ENVIRONMENTAL CONDITIONS

by

LUIS MARIA B. GARCIA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **TESTOSTERONE AND ESTRADIOL-17 BETA PRODUCTION BY GOLDFISH, *CARASSIUS AURATUS*, OVARIAN FOLLICLES *IN VITRO*: INFLUENCE OF SEASON AND ENVIRONMENTAL CONDITIONS** submitted by **LUIS MARIA B. GARCIA** in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

ABSTRACT

The effects of time of day, season and the length of acclimation to different environmental conditions on *in vitro* ovarian follicular steroidogenesis in the goldfish, *Carassius auratus*, were investigated. Sexually maturing and mature goldfish were initially exposed to a 12 h light: 12 h dark at 12°C (12L: 12D / 12°C) for 8 days and then to a 16L:8D / 20°C or a 12L: 12D / 12°C experimental condition for various lengths of time in "winter" (January to March) and in "spring" (April to July). After the designated number of days of acclimation to these experimental conditions, fish were sampled diurnally and the ovarian follicles were mechanically dispersed and passed through several sieves of decreasing mesh sizes to acquire three size classes of follicles at different developmental stages: large-sized follicles (diameter greater than 800 microns) consisted largely of tertiary yolk globule oocytes; intermediate-sized follicles (diameter between 350 microns and 800 microns) were largely oocytes at late yolk vesicle and primary / secondary yolk globule stages; and small-sized follicles (diameter less than 350 microns) were mostly oocytes at early and middle yolk vesicle stages. Aliquots of the pooled dispersed follicles from each size group were incubated for 4 hours at their acclimation temperature of 12°C or 20°C in buffered medium alone (control) or medium with varying doses of carp gonadotropin (0.01-1.0 microgram GTH / ml). Testosterone (T) and estradiol-17 beta (E_2) in the incubation medium were measured by radioimmunoassay and the results were expressed as picograms (pg) of steroid per milligram (mg) of protein, per unit of follicular surface area (mm^2) and per follicle.

After 5-7 days of exposure to 16L:8D / 20°C, GTH-treated large, intermediate and small follicles from "winter" (January) fish had greater T and E_2 production per mg of protein at 1100h than at 1900h, whereas only large follicles from "spring" (April) fish were more responsive to GTH at 1900h than at 1100h. Hence, the data support previous suggestions that teleost ovaries exhibit a temporal variation, on a daily basis, in response to GTH stimulation.

Steroidogenesis in large follicles from sexually recrudescing fish in "winter" (January-February) is promoted by GTH after a short-term (5-7 days) and a long-term

(30-32 days) exposure to long photoperiod and warm water conditions (16L:8D / 20°C). In contrast, the progressive decline in T and E_2 production by large follicles in "spring" (May-June) may signal a shift in ovarian follicular production of T and E_2 to progestogens appropriate for ovulation and spawning of large preovulatory follicles, or it may indicate a decline in responsiveness to GTH stimulation resulting from regression or atresia of large follicles in "spring".

Following a long-term (30-32 days) acclimation of goldfish to 16L:8D / 20°C in "winter", intermediate follicles had a constant level of steroidogenic responsiveness to GTH stimulation in terms of E_2 production per unit surface area and per follicle. However, there was a decline in E_2 output per unit surface area and per follicle by GTH-treated intermediate follicles after 30-32 days of acclimation to long photoperiod and warm water conditions in "spring". On the other hand, the levels of E_2 attained by each intermediate follicle treated with GTH in "winter" and in "spring" were similar until Days 11-13, indicating that intermediate follicles at the middle stages of vitellogenesis maintain a constant level of responsiveness to GTH to maintain their development.

The relative contribution of intermediate and small follicles to the circulating pool of T and E_2 in female goldfish subjected for 5-7, 11-13 or 30-32 days at 16L:8D / 20°C in "winter" is greater than by the more responsive large follicles; however, T and E_2 in circulation under similar environmental conditions in "spring" is due to an approximately equal contribution by large and intermediate follicles.

A low level of responsiveness to GTH stimulation characterized the ovarian follicular steroid production from goldfish exposed for different lengths of time at 12L:12D / 12°C in "winter" (March) and in "spring" (June-July). Follicular incubation at 12°C with GTH either failed to stimulate T and E_2 production by large and intermediate follicles or steroid production levels under GTH treatment at Days 6-8 and 30-32 were low. A large proportion of the circulating T and E_2 in March, and also possibly in June-July at 12L:12D / 12°C, may be attributed to the relatively more abundant but less responsive intermediate follicles. The low production of T and E_2 by ovarian follicles incubated with GTH at 12°C, in contrast to levels produced at 20°C incubation, suggests that the rate of follicular steroidogenesis is suppressed under cold water conditions.

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I. INTRODUCTION

In teleost fishes in the temperate zone, the period of reproductive activity is confined to certain seasons of the year. The interactions of the fishes' inherent biological rhythms and the environment are thought to govern these reproductive events (Crim, 1982). Hence, gonadal development and maturation each proceed in response to a set of cues perceived by the peripheral and central nervous systems. Since these cues occur in a very periodic and precise manner, the responsiveness of the teleost's gonads to gonadotropin (GTH) hormone might also have some synchrony with the existing set of environmental factors.

Much of the information on fish reproduction deals with investigations correlating the onset of gonadal maturation with plasma levels of GTH released from the pituitary. Generally, in female salmonids and cyprinids the plasma concentrations of GTH gradually increase during the period of gonadal recrudescence (Peter and Crim, 1979; Peter, 1981). At the time of oocyte maturation and ovulation a surge of GTH secretion occurs in the goldfish, *Carassius auratus* (Stacey *et al.*, 1979), the white sucker, *Catostomus commersoni* (Stacey *et al.*, 1984), and the rainbow trout, *Salmo gairdneri* (Fostier *et al.*, 1978; Scott *et al.*, 1983).

The annual profiles of androgens and/or estradiol-17 beta (E_2) have also been investigated in a variety of teleost fish species,including the brown trout, *Salmo trutta* (Kime and Manning, 1982), male and female rainbow trout, (Lambert *et al.*, 1978; Scott *et al.*,1980, 1983; Schmidt and Idler, 1982; Schreck *et al.*, 1973), Atlantic salmon, *Salmo salar* (Hunt *et al.*, 1982), Indian catfish, *Heteropneustes fossilis* (Lamba *et al.*, 1983), plaice, *Pleuronectes platessa* (Wingfield and Grimm, 1977), winter flounder, *Pseudopleuronectes americanus (Campbell et al.*, 1976), amago salmon, *Oncorhynchus rhodurus*, (Kagawa *et al.*, 1983a), white-spotted char, *Salvelinus leucomaenis* (Kagawa *et al.*,1981), striped mullet, *Mugil cephalus* (Dindo and MacGregor, 1981), and goldfish, (Schreck and Hopwood, 1974; Kagawa *et al.*, 1983b). In general, corresponding to the initial rise of GTH and the commencement of the initial stages of oocyte growth and development, there is a gradual increase in plasma levels of testosterone (T) and E_2.

Histochemical and ultrastructural studies indicate that the granulosa cell layer and the special theca cells surrounding the oocytes are the major cellular sources of steroids in teleost ovaries (Hoar and Nagahama, 1978; Nagahama, 1982). *In vitro* investigations on isolated ovarian follicles of the amago salmon by Kagawa *et al.*(1982) have shown that the granulosa cell layer synthesizes estrogens from testosterone produced from special theca cells. This mode of production follows the two-cell type model for follicular estrogen synthesis in mammals (Dorrington and Armstrong, 1979).

During the period of oocyte growth in the white-spotted char and the amago salmon, there is a good correlation of the plasma levels of T, E_2 and progesterone with the *in vitro* production of these steroids by isolated ovarian follicles in response to GTH stimulation (Kagawa *et al.*, 1981, 1983a; Nagahama and Kagawa, 1982). In both species, the ability to produce T , E_2 and progesterone by ovarian follicles *in vitro* also varies seasonally in correlation with the seasonal changes in plasma levels of T, E_2 and progesterone. However, around the time of oocyte maturation and ovulation, changes in steroid production occur. Preovulatory follicles produce more T and E_2 than post-ovulatory follicles; post-ovulatory follicles produce higher progestagen levels (notably 17 alpha,20 beta-dihydroxy-4-pregnen-3-one or 17 alpha, 20 beta-P) whereas preovulatory follicles produce little or no progestagens. This pattern in the production of T and E_2 has been taken as evidence for a decrease in aromatase enzyme activity in the granulosa cell layer immediately prior to ovulation (Young *et al.*, 1983).

In two previous studies on rainbow trout, Fostier *et al.*(1978) and Scott *et al.*(1983) found a decrease in plasma levels of T and E_2 immediately before ovulation, at the same time that GTH and 17 alpha, 20 beta-P plasma levels were simultaneously increasing. In contrast, in goldfish undergoing spontaneous or brain-lesion induced ovulation, plasma GTH and E_2 levels were both high at the same time (Stacey *et al.*, 1983); plasma testosterone levels peaked early in oocyte maturation and had already decreased a few hours later at the time of ovulation. A possibility which was put forward to explain the trend for increasing levels of steroids (T, E_2, 17 alpha, 20 beta-P) and GTH in cyprinids at the time of final oocyte maturation is that these species have asynchronous oocyte growth; the GTH surge during ovulation in cyprinids stimulates immature or non-preovulatory follicles to produce high amounts of T and E_2 (Stacey *et al.*, 1983;

Kagawa *et al.*, 1983b).

The coincident rise in the seasonal levels of T and E_2 with GTH in many fish species has led investigators to speculate that indeed these steroids may mediate, and possibly control, many of the GTH-dependent reproductive processes, as in the case of other vertebrates (Chester Jones *et al.*, 1972). To illustrate, estrogens are known to induce the synthesis and secretion of yolk proteins by the liver in the rainbow trout (van Bohemen *et al.*, 1982; Idler and Campbell, 1980) and in the Indian catfish (Sundararaj and Nath, 1981); also, high levels of estrogens have been correlated with high plasma levels of vitellogenin. However, the role of testosterone in female teleosts is not entirely clear. High plasma T levels may be associated with final oocyte maturation and spawning in the amago salmon (Young *et al.*, 1983b) in terms of stimulating the accumulation of pituitary GTH prior to the rapid increase in GTH secretion and the surge of plasma levels during ovulation and spawning.

Aside from the descriptions showing the annual profiles of reproductive hormones, several investigators have demonstrated the presence of short-term patterns of hormone fluctuations in several teleosts. de Vlaming and Vodicnik (1977a) found a daily variation in pituitary GTH content in the golden shiner, *Notemigonus crysoleucas*, exposed to long photoperiod (15.5 h light:8.5 h dark) at 15°C, with GTH being low early in the light phase and high late during the light phase. Breton *et al.* (1972) demonstrated a mid-day peak (1100h) in plasma GTH of sexually mature female goldfish held under natural environmental conditions in July; however, GTH levels in mature male goldfish remained constant throughout the day. Recently, Hontela and Peter (1978) showed the presence of significant daily variations in the serum GTH levels of female goldfish acclimated to several combinations of temperature and photoperiod for a maximum of 9 days. Significant daily peaks in GTH occurred in recrudescing and mature fish whereas sexually regressed fish showed little or no circadian variations. Hontela (1982) further demonstrated the occurrence of significant fluctuations of serum GTH in female goldfish at two different times during the breeding season. Goldfish subjected to 16L:8D / 20°C had mid-day (1100h) GTH surges after 5-7 days in November and 11-13 days in March; relatively high and constant daily levels of serum GTH were detected after a month under these conditions in both November and March. Significant daily fluctuations were observed in

fish kept for 32 days on 12L:12D / 12°C in March but not in November. Interestingly, goldfish with atretic oocytes did not have fluctuations of serum GTH levels. On the basis of these results, Hontela and Peter (1978) and Hontela (1982) suggested that the existence of daily GTH surges in the goldfish and in other fish species may be of importance in stimulating gonadal development.

The rate and timing of reproductive development in teleosts is influenced by variations in environmental conditions which operate through the hypothalamo-pituitary-gonadal axis by mechanisms which are not fully understood. For some teleost species, temperature and / or photoperiod largely controls the onset of reproductive activity. Warm temperatures (20-22°C) stimulated gonadal growth and maturation and increased the number of spawning periods in the tench, *Tinca tinca* (Breton *et al.*, 1980). Also, vitellogenesis in tench was accelerated only if the mean daily temperature was more than 10°C. On the other hand, salmonids respond primarily to variations in photoperiod to cue their reproductive cycles (Peter and Crim, 1979; Peter, 1981). Trout exposed to a shortening photoperiod regime under both high and low temperatures showed enhanced spermatogenesis; however, plasma GTH levels under high temperatures and a short photoperiod regimes were higher than under low temperatures and short photoperiod conditions (Breton and Billard, 1977). The interaction of temperature and photoperiod would therefore seem to influence the release of pituitary GTH via the hypothalamus. In the goldfish, a long-term exposure to warm water conditions is associated with elevated plasma GTH but gonadal recrudescence is largely inhibited (Gillet *et al.*, 1977, 1978).

Since the secretion of GTH and other reproductive hormones follows both a seasonal and a daily cycle corresponding to variations in the environment, it follows that these cycles and, indirectly, the environment may influence the responsiveness of endocrine organs, including the gonads. In the golden shiner maintained on a 15.5L:8.5D / 15°C regime, salmon GTH stimulated the rate of gonadal development if injected early, but not late, during the photophase (de Vlaming and Vodicnik, 1977). Also, the anti-gonadal effect of injections of prolactin in the golden shiner was evident only if the injections were done early in the light phase (de Vlaming and Vodicnik, 1977). Ovarian fragments from mature striped mullets maintained for 45 days at 12L:12D / 21°C likewise

showed a circadian rhythm of oocyte responsiveness to GTH and prostaglandin treatments in terms of oocyte accumulation of cyclic adenosine 3',5'-monophosphate (cAMP) (Kuo and Watanabe, 1978). Similarly, female goldfish acclimated to 8L:16D / 14-15°C were found to have a temporal variation in responsiveness to GTH in terms of GSI (gonadosomatic index) and oocyte development; daily injections for 14-18 days with a low dosage of salmon and carp GTH at 4 and 8 hours after light onset were effective in stimulating an increase in GSI, whereas injections at the onset of light were ineffective (Peter *et al.*, 1982). Also, female goldfish held under a long photoperiod condition (16L:8D) and 14-15°C showed an increased GSI in response to daily injections of a low dose of carp GTH at only 12 hours after light onset. Taken together, these investigations suggest that the temporal responsiveness of teleost gonads to several trophic hormones results from fluctuating levels of endogenous GTH in phase with certain environmental cues.

Very little attention has been paid, however, to the effects of acclimation to different conditions together with the trophic influences of GTH and other hormones on the steroidogenic capacity of the gonads. Along this line, Kime (1982) demonstrated *in vitro* that increasing temperatures favor testicular and hepatic synthesis of steroid glucuronides and an increased rate of exogenous substrate utilization in the fish testes.

In the present study, the hypothesis that environmental conditions affect the capacity of the teleost gonads to synthesize T and E_2 was tested. More specifically, the effect of length of acclimation of female goldfish to two sets of environmental regimes and the effect of incubation temperature on steroid production by ovarian follicles *in vitro* were examined. By testing the steroid secretion response *in vitro* of dispersed ovarian follicles from goldfish, the seasonal changes in responsiveness to GTH by different sizes of follicles were investigated.

II. MATERIALS AND METHODS

A. Experimental Animals

Goldfish, *Carassius auratus*, of the common or comet varieties were purchased from Grassyfork Fisheries (Martinsville, Indiana) or from Ozark Fisheries (Stoutland,Missouri) in December, February, March and May. The fish were sexed upon arrival by gently squeezing the abdomen and noting if any yolky oocytes are extruded; fish for these experiments were either sexually maturing (undergoing ovarian recrudescence) or in a mature (preovulatory) stage. The fish were held in a 4800 l capacity flow-through tank in the main room of the aquatic facilities of the Department of Zoology for a period of 2-4 weeks prior to use in the experiments (laboratory acclimation period) and were fed twice daily to excess with Ewos trout pellets (Astra Chemical Ltd., Mississauga, Ontario). The temperature was regulated at 12-14 ^{0}C and room lights simulated the natural photoperiod (Edmonton, Alberta).

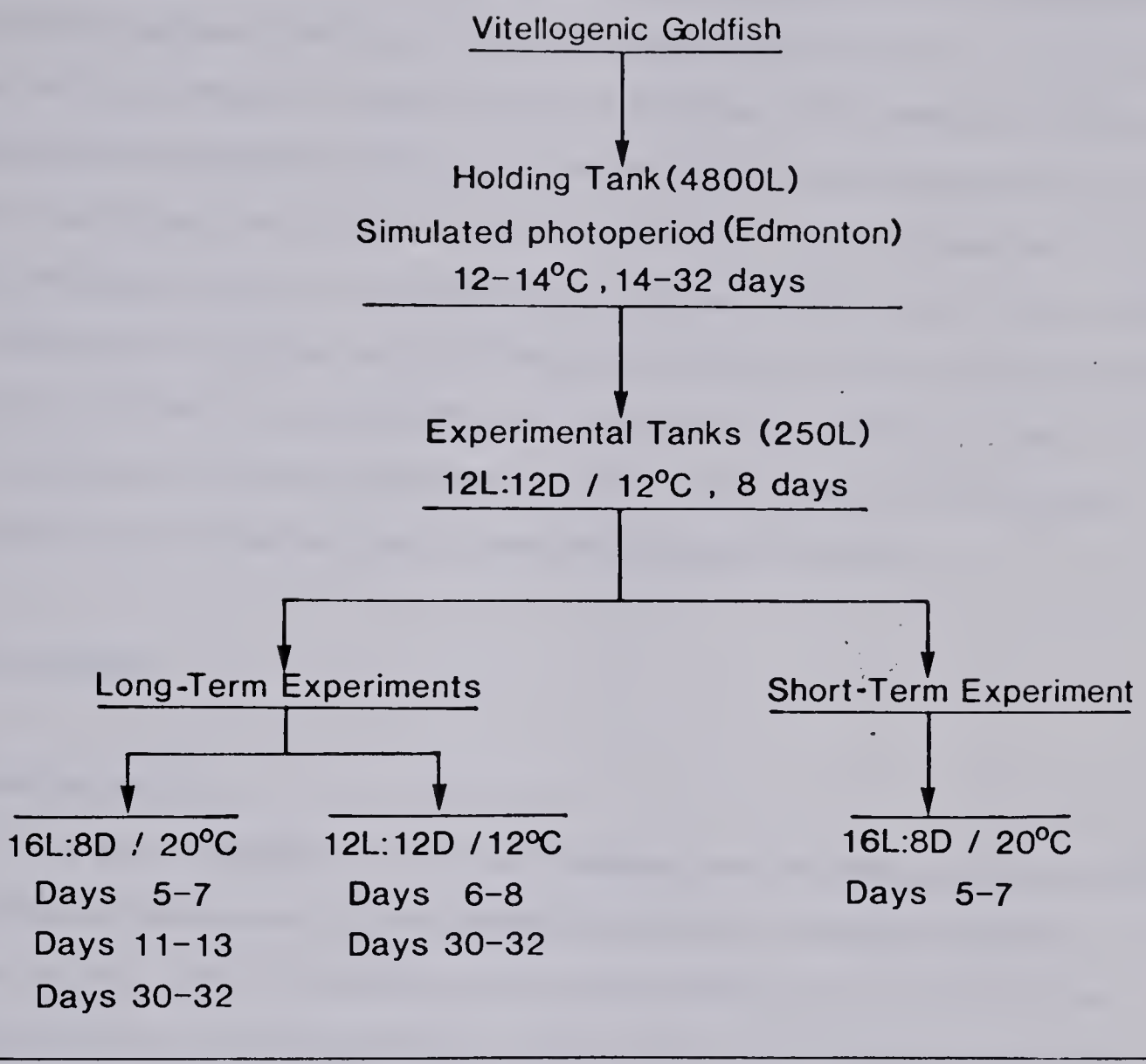
B. Experimental Maintenance Regimes

1. Experimental Protocol

To assess seasonal changes in ovarian steroidogenic capacity goldfish were sampled during the period from January to March of 1983, designated as "winter" fish, and during April to July of 1983, designated as "spring" fish.

The protocol of the experiments is outlined in Fig. 1. Following a laboratory acclimation period of 2-4 weeks in a 4800 l tank, under 12-14^{0}C and a simulated natural photoperiod (Edmonton), fish were transferred to 250 l flow- through experimental tanks and held on a 12L:12D (lights on at 0600h) photoperiod and a temperature of 12± 1^{0}C (designated the initial environmental regime). On Day 1, eight days after the 12L:12D/ 12^{0}C regime was begun, the experimental environmental regimes of 16L:8D/20^{0}C were imposed, or 12L:12D/ 12^{0}C was continued. Light was provided by a 15 watt cool white fluorescent lamp in each experimental tank. Feeding was at 4 hours (1000h) and 10 hours (1600h) after lights were automatically switched on.

Figure 1. An outline of the experimental protocol and sampling schedules used in the study.



SAMPLING SCHEDULE

Long-Term Experiments

First Day : 0700H
1900H

Second Day : 0300H
1500H

Third Day : 1100H
2300H

Short-Term Experiment

First Day :
Second Day :
Third Day :
1100H
1900H

2. Sampling Schedule

Following the protocol outlined in Fig. 1, fish were sampled over three consecutive days after the designated numbers of days of acclimation on the experimental environmental regimes. In the Short-Term Experiments, fish were held on a 16L:8D / 20°C regime, and were sampled on Days 5, 6 and 7 at 1100h and 1900h each day. Hontela (1982) had shown significant differences in the serum GTH levels at these particular times of day in female goldfish subjected to 16L:8D / 20°C for 5-7 days in December. For the Long-Term Experiments, fish were sampled at 0700h and 1900h on the first day, 0300h and 1500h on the second day, and 1100h and 2300h on the third day. In the Long-Term Experiment, fish held on a 16L:8D / 20°C were sampled on Days 5,6 and 7, Days 11, 12 and 13, and Days 30, 31 and 32. Fish on a 12L: 12D / 12°C regime in the Long-Term Experiments were sampled on Days 6,7 and 8 and Days 30, 31 and 32.

C. Tissue Culture

1. Oocyte Dispersion

After spinal transection, the fish were weighed, and the ovaries of five to ten fish dissected out and placed in a 300 ml-capacity specimen dish containing a modified Cortland's buffer solution. The buffer solution was modified from Wolf (1963), and contained 0.05%(w / v) bovine serum albumin (BSA) and 0.1%(v / v) Gentamycin sulfate (Sigma Chemicals Company, St. Louis, Missouri). The total ovary weight was recorded and the gonadosomatic index (GSI = ovary weight / total body weight x 100) calculated. All dispersions were carried out in finger bowls over chipped ice.

The pooled ovaries were gently teased into clumps of 25-50 vitellogenic oocytes with a pair of fine forceps, with frequent changing of the buffer solution. Clumps of oocytes were subsequently dispersed into individual follicles by gently aspirating the clumps of teased oocytes in and out of a blunt-tipped (4 mm inside diameter) disposable polyethylene pipette (Bio-Rad Laboratories, Mississauga, Ontario) in order to separate the individual follicles. The dispersed follicles were then sorted through three layers of sieve cloth. The first layer (mesh size 1300 microns) removed clumps of ovarian tissue and clumps of non-dissociated follicles, while the second (mesh size 800 microns) and third

(mesh size 350 microns) layers separated follicles into three size categories: large follicles (diameter greater than 800 microns), intermediate follicles (diameter between 350 microns and 800 microns) and small follicles (diameter less than 350 microns). The three follicle size categories were then each pooled into 12x75 mm culture tubes and centrifuged at 500 rpm (50xg) for 5 min at 4°C. The supernatant was discarded and the oocytes were thoroughly mixed by aspirating gently with a blunt-tipped polyethylene pipette.

2. Follicle Incubation

The dispersed follicles were distributed in 0.1 ml aliquots, with an automatic hand pipettor, into 12x75 mm glass culture tubes in a random manner; each tube contained either 0.5 ml of Cortland's buffer alone (controls) or varying doses of carp gonadotropin (cGTH, a gift from Dr. B. Breton, Laboratoire de Physiologie des Poissons, Institut National de la Recherche Agronomique, Campus de Beaulieu, Rennes, France) within the range of 0.1 to 1.0 micrograms / ml buffer. The cGTH used in the experiments, prepared as described in Peter *et al.*(1982), was the same as the high carbohydrate content "maturational" GTH (Con A-II) that stimulates cAMP levels in ovaries, steroid synthesis and secretion, oocyte maturation and ovulation, and spermiation (Idler and Ng, 1979; Ng and Idler, 1979). Each dose treatment in an experiment was tested in at least three replicates. Tissue incubations were carried out in air, either in a shaking water bath maintained at 12°C for follicles from fish acclimated at 12°C or a mechanical shaker at room temperature (20°C) for follicles from fish acclimated at 20°C. A pilot time course experiment performed on follicles from fish acclimated at 16L:8D / 20°C indicated that maximal levels of steroids were released into the medium within 4-6 hours. A time course experiment of steroid production by ovarian follicles from goldfish acclimated to 12L:12D / 12°C was not performed. For practical purposes, all follicular incubations were carried out for only 4 hours. At the end of the incubation period, each culture tube was centrifuged for 5 min at 500 rpm (50xg) and 4°C, the supernatant pipetted into a 1.5 ml centrifuge tube and stored at -20°C until assayed for steroids.

3. Incubation Temperature Experiment

To determine the effect of incubation temperature on steroid production, follicles from fish held for 8 days at 16L:8D / 20°C or 12L:12D / 12°C, after the initial acclimation period, were incubated at 12°C and 20°C for 4 hours and following the procedure described elsewhere (see RESULTS for details).

D. Radioimmunoassay of Testosterone and Estradiol-17 Beta In the Incubation Medium

1. Reagents and Chemicals

a. Chemicals

All chemicals and reagents used in the extraction and chromatography were of spectra-analytical grade and were purchased from Fisher Scientific, Inc. (Don Mills, Ontario) or BDH Chemicals (Toronto, Ontario).

b. Assay Buffer

The assay buffer consisted of 0.05 M Tris-HCl (Trizma Compound 8.3, pH 8.3 at 25°C, Sigma Chemicals Co.) containing 0.1 M NaCl, 0.1% sodium azide and 0.1% gelatin.

c. Steroid Standards

Testosterone (delta^4-androsten-17 beta-ol-3-one) and estradiol-17 beta [1,3,5,(10)-estratrien-3,17 beta-diol] were purchased from Sigma Chemicals, Inc.. Stock solutions [200 nanograms(ng) / ml] were prepared in absolute methanol and stored at -20°C. Aliquots from this stock solution were serially diluted with the assay buffer to acquire the final concentrations of the standard solutions.

d. Antisera

Sealed vials of freeze-dried rabbit anti-testosterone-7 alpha-BSA serum and rabbit anti-17 beta-estradiol-6-BSA were purchased from Miles Laboratories, Ltd. (Rexdale, Ontario). The antisera were reconstituted with 5 ml of assay buffer prior to use and this served as the stock antiserum solution. A 1:10 dilution of the stock solution yielded the working antiserum solution, which gave 30-50% binding of the radioactively labelled steroids in the radioimmunoassay.

The cross reactions of related steroids with the antisera were provided by the manufacturer (Tables 1 and 2).

e. Radioactive Steroids

(1,2,6,7-^{3}H)Testosterone (approximately 100 Curies(Ci) / mmol) and (2,4,6,7-^{3}H)Estradiol-17 beta (approximately 100 Ci / mmol) were purchased from Amersham Radiochemical Corporation (Oakville, Ontario). Each radioactive steroid was first diluted to 25 microCi / ml with benzene:absolute ethanol (9:1), and further to 5 microCi / ml with absolute ethanol. One milliliter aliquots of 5 microCi / ml were transferred to 1.5 ml centrifuge tubes and stored at -20^{0}C.

Before use in a radioimmunoassay, the 5 microCi / ml aliquots of the labelled steroids were dried under nitrogen and redissolved in assay buffer solution to get two working solutions of labelled steroid, having approximately 1000 counts per min(cpm) / 25 microliter in the extraction and chromatography procedures, and approximately 10000-15000 cpm / 100 microliter for the RIA.

f. Charcoal Suspension

The charcoal suspension consisted of 0.5%(w / v) Norit A activated charcoal and 0.05%(w / v) Dextran T-70 in assay buffer solution.

2. Extraction Procedure

Incubation medium (0.5 ml) was transferred to duplicate extraction tubes together with 25 microliter of tracer (approximately 1000 cpm) by using accurate measuring devices (Oxford pipettor) with disposable tips. The tubes were vortexed and extracted twice with 1 ml each of diethyl ether and centrifuged at 2000 rpm (900xg)for 5 min. The aqueous phase was frozen over dry ice and was pooled in another centrifuge tube. The pooled extracts were dried under a stream of nitrogen in a 60^{0}C water bath and were subsequently concentrated with 1 ml of absolute ethanol prior to chromatography (see below). Likewise, 25 microliters of tracer alone were transferred directly to scintillation vials to obtain a total count of radioactivity for calculation of percentage recovery.

To eliminate the extraction procedure prior to radioimmunoassay of the incubation medium from all the experiments with follicles, five to seven samples with increasing amount of steroids dissolved in the incubation medium were run in duplicate through the

Table 1. The relative cross-reactivity of various steroids for the antibody (anti-Estradiol-17 beta-6-BSA serum) used in the Estradiol-17 beta radioimmunoassay. Data were provided by the manufacturer (Miles Laboratories, Ltd.).

STEROID	% CROSS-REACTION
Estradiol-17 beta	100.0
Estrone	2.2
Estriol	0.56
Estradiol-17 alpha	1.1
6 alpha-Hydroxy-estradiol-17 beta	62.0
6-Keto-estradiol-17 beta	52.0
17 alpha-Ethynyl-estradiol-17 beta	<0.01
Equilenin	1.3
Testosterone	<0.01
Progesterone	<0.01

Table 2. The relative cross-reactivity of various steroids for the antibody (anti-Testosterone-7 alpha-BSA serum) used in the Testosterone radioimmunoassay. Data were provided by the manufacturer (Miles Laboratories, Ltd.).

STEROID	% CROSS-REACTION
Testosterone	100.0
5 alpha-Dihydrotestosterone	17.0
5 beta-Dihydrotestosterone	5.0
17 alpha-Epitestosterone	0.8
Androstenedione	6.0
5 alpha-Androstane-3,17-dione	<0.001
Dehydroepiandrosterone	<0.001
5 alpha-Androstane-3 beta,17 beta-diol	<0.001
11-Oxotestosterone	4.4
Progesterone	<0.001
Cortisol	<0.001
Estradiol-17 beta	<0.001

radioimmunoassay without extraction and compared with the same set of samples which had been extracted, as described above. The percentage recovery of the samples was recorded and the results analyzed by linear regression analysis. Since the radioimmunoassay values of extracted and un-extracted samples agreed well with correlation coefficients (*r*) of 0.96 and 0.99 ($P<0.05$) for E_2 and T, respectively (Table 3), it was deemed not necessary to extract the samples prior to radioimmunoassay.

3. Celite Chromatography

Since antibodies raised against steroid hormones cross-react to different degrees with other related steroids, the use of these antibodies as detection methods may require a chromatographic separation and purification procedure of the steroid hormone prior to radioimmunoassay, to maintain the specificity of the measurement.

The chromatographic procedure used here followed that outlined by Abraham *et al*.(1972), with minor modifications. Celite (Diatomaceous Earth, Sigma Chemicals Co.) was prepared by heating in an oven at 540°C overnight to remove water and any interfering organic material. The celite was cooled in a dessicator for a few hours. Prior to packing, dried and baked celite was mixed with ethylene glycol:propylene glycol (50:50) for T and ethylene glycol alone for the E_2 partition chromatography at a ratio of 1 ml per 2 gm celite. Then, it was packed into disposable 5 ml glass pipettes (inside diameter 0.5 cm) to a height of 5 cm; a small glass bead was added to the bottom of each pipette to retain the celite. The columns were washed twice with 3.5 ml of isooctane before use.

After extraction, the 1 ml extracts were dried under nitrogen in a 60°C water bath and reconstituted with 1 ml of isooctane. The reconstituted extracts were then applied to the columns using disposable Pasteur pipettes, and eluted under nitrogen pressure to obtain a flow rate of 20-30 drops per minute.

For testosterone, elution was carried out in a stepwise pattern using 3.5 ml each of isooctane, isooctane:ethyl acetate (90:10), and isooctane:ethyl acetate (80:20). The T fraction was contained in the last 3.5 ml fraction. Likewise, E_2 was eluted by successively applying 3.5 ml each of isooctane, isooctane:ethyl acetate (85:15), and isooctane:ethyl acetate (60:40) to the column. E_2 was contained in the last 3.5 ml fraction.

Table 3. The recovery of increasing steroid standards added to the incubation medium and their relationship between steroid levels measured in extracted incubation medium versus media without extraction and in media which were extracted then chromatographed versus media without extraction and chromatography. *n* = number of samples assayed.

	n	% Recovery	Slope	Correlation Coefficient (*r*)
ESTRADIOL-17 BETA:				
Extracted	7	51.5-93.9	0.99	0.96
Extracted and Chromatographed	7	45.0-61.1	1.07	0.99
TESTOSTERONE:				
Extracted	5	70.1-93.7	1.01	0.99
Extracted and Chromatographed	7	58.8-90.6	0.81	0.99

The T and E_2 fractions were subsequently dried under nitrogen, reconstituted with 1 ml of absolute ethanol, and a small measured aliquot taken to calculate the total percentage recovery. Then, another measured aliquot was taken, dried under nitrogen, and subsequently reconstituted with 0.1 ml of assay buffer for radioimmunoassay.

Seven samples of increasing amounts of T and E_2 added to the follicle incubation medium were run in duplicate directly through the radioimmunoassays, and another similar set of seven samples was extracted and chromatographed following the method described before being run through the radioimmunoassay. The total percentage recovery of each sample was recorded, and the steroid levels analyzed by linear regression analysis. Since the radioimmunoassay values of the extracted and chromatographed samples and the directly assayed samples agreed well (r = 0.99 for both steroids, $P<0.05$), it was deemed not necessary to have the incubation medium samples extracted and chromatographed prior to radioimmunoassay for E_2 and T (Table 3).

Several dilutions of the incubation medium were assayed and the dilution curves compared with standard T and E_2 in order to ascertain whether the T and E_2 secreted *in vitro* by goldfish ovarian follicles were immunologically similar to the standard steroids used in the RIA's. Displacement curves by several diluted incubation media and by increasing amounts of unlabelled steroids were statistically tested by ANCOVA (Analysis of Covariance) (Snedecor and Cochran, 1980) and were parallel($P>0.01$)

4. Radioimmunoassay Procedure

A range of concentrations of the steroid standards (0,0.075, 0.15, 0.31,0.63,1.25,2.5,5.0 ng/ml) for both the E_2 and T RIA's were pipetted (0.1 ml) in quadruplicate into polypropylene plastic culture tubes (12 x 75 mm). Quadruplicate samples were used to obtain more representative values of the steroid standards. Duplicate sets of tubes contained 0.1 ml samples from the follicle incubation experiments. Labelled steroids (approximately 10000-15000 cpm in 0.1 ml) were added to each tube. Except for the total count tubes and blank (non-specific binding) tubes, 0.5 ml of the working antibody solution was added to each tube; 0.5 ml of assay buffer instead of the antibody was added to each of the total count and blank tubes. Therefore, prior to the addition of the charcoal suspension, all tubes had a total volume of 0.7 ml each.

After the addition of the antibody, all tubes were incubated overnight (16-18 hours) at room temperature, after which all tubes were cooled to 4°C for at least 15 minutes. Then, 0.2 ml of the charcoal suspension was added to each tube, except for the total count tubes to which 0.2 ml of assay buffer was added instead. All assay tubes were shaken for 1 min and further incubated at 4°C for 10 min. All tubes, except for the total count tubes, were centrifuged at 4°C at 2100 rpm (900xg) for at least 15 min. Finally, a 0.5 ml aliquot of the supernatant from each tube was carefully pipetted to plastic scintillation vials and suspended in 15 ml of scintillation cocktail (Toluene, 70%; 2-methoxyethanol, 30%; POPOP(1,4-Bis[2-(5-phenyloxazolyl)]-Benzene), 0.01%; PPO (2,5-Diphenyloxazole), 0.4%). Samples were counted in a Beckman liquid scintillation spectrometer (Model LS9000) with 40-50% counting efficiency for tritium.

The intra-assay coefficient of variation (%CV) or the variability of a single sample counted in a single assay run (%CV = SD / mean x 100) was 9.7% (n=4) and 6.5% (n=4) for the T and E_2 RIA's, respectively. Inter-assay %CV or the variability of a sample counted in several assay runs was 13.1% (n=20) and 8.1% (n=18) for the T and E_2 RIA's, respectively. The sensitivity (or the least significant steroid level detectable from zero) for both assays was 0.04 ng / ml or 4 pg / tube.

E. Determination of Oocyte Number, Oocyte Diameter and Oocyte Histology

After the 4 hour follicle incubation period, several sets of incubation tubes each containing the three sizes of follicles were routinely counted under a dissecting microscope. The number of large, intermediate and small follicles in each incubation tube is shown in Table 4. The diameter of the follicles was also measured with an ocular micrometer.

Samples of dispersed follicles were fixed in Bouin's solution following incubation, and standard paraffin embedding, and hematoxylin and eosin staining procedures used. The number of oocytes in each stage was counted for the different size ranges of follicles used in the experiments (Table 5) by using the classification of Yamazaki (1965) for the different ovarian stages of the goldfish. The proportions of each oocyte stage for each of the follicle size categories remained relatively constant in ovarian dispersions performed from January to July.

Table 4. The number of follicles and the total protein content of each incubation tube for each of the three follicle sizes separated from goldfish ovaries.

Follicle Size	Number of Follicles / tube (mean±SEM, *n*)	Milligrams of Protein / tube (mean±SEM, *n*)
Large (diameter greater than 800 microns)	245.0±9.0 (38)	7.5±0.17 (14)
Intermediate (diameter between 350 microns and 800 microns)	575.0±18.0 (39)	5.1±0.15 (19)
Small (diameter less than 350 microns)	1671.0±52.0 (30)	4.3±0.17 (26)

Table 5. The cellular composition of the three follicle sizes separated from goldfish ovaries.

Follicle Sizes	Oocyte Stages[1]					
	Yolk Vesicle			Yolk Globule		
	Early	Middle	Late	Primary	Secondary	Tertiary
Large (diameter greater than 800 microns)			0.21±0.04[2]	0.22±0.05	0.24±0.03	1.30±0.05
Intermediate (diameter between 350 microns and 800 microns)		0.47±0.06	0.95±0.05	0.98±0.06	0.29±0.04	
Small (diameter less than 350 microns)	1.02±0.06	0.86±0.04	0.47±0.02			

[1] Based on oocyte classification by Yamazaki (1965).
[2] Expressed as oocyte stage per mm^2 of visual field at 40X from 5-8 observations (mean±SEM).

F. Protein Assay

To 0.1 ml of the dispersed follicles was added 1 ml of 0.5 N NaOH and the follicles subsequently digested in a 60°C water bath. Four milliliters of double distilled water was added after digestion and a 0.1 ml aliquot taken for the protein assay following the procedure outlined by a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories). Bovine serum albumin (BSA) was used as the protein standard. The total amount of protein present in each incubation tube for each of the three sizes of follicles is shown in Table 4 and, similarly the protein content remained relatively constant for each follicle size category dispersed from ovaries in January to July.

G. Data Presentation and Statistical Analyses

The results of the present investigation were presented in three ways: pg of steroid per mg of protein, pg of steroid per unit surface area of follicle (mm^2) and pg of steroid per follicle. The steroid production per mg of protein was calculated by dividing the total amount of steroid measured in each incubation tube by the mean protein content present in each tube for each of the three size classes of follicles. The mean follicular diameter of each follicle size class and the mean number of follicles present in each incubation tube was used to calculate an average follicle surface area prior to expressing the data as pg of steroid per unit surface area. Steroid production per follicle was calculated by dividing the total amount of steroid in each incubation tube by the mean number of follicles in each tube for each of the three follicle sizes.

In the Long-Term Experiments, variability of the results and low sample sizes (n) precluded comparisons between times of day; therefore, the results for the three consecutive days of sampling were pooled to allow comparisons between doses of GTH within experiments and length of acclimation between experiments.

Unpaired t-test ($P<0.05$) was used to compare differences between values from the same treatment group and differences between their respective controls. Analysis of variance (ANOVA) followed by Duncan's Multiple Range Test at $P<0.05$ was used to determine differences between mean values expressed in pg steroid per unit surface area or pg steroid per follicle at different sampling days over the 32-day experimental period (Snedecor and Cochran, 1980).

III. RESULTS

A. Short-Term Experiments

1. January, 1983

Fish were subjected to 16L:8D / 20°C for a maximum of 7 days subsequent to 8 days at 12L:12D / 12°C in January, 1983. The results are presented in Figs. 2 and 3 for follicles sampled from these "winter" fish at two different times of the day for three consecutive days.

Levels of testosterone (T) in the incubation medium for all the three follicle sizes were non-detectable when the follicles were incubated with buffer alone; however, GTH caused a dose-dependent increase in T production per mg of protein by all the three sizes of follicles (Fig. 2). Based on steroid production per mg of protein, large follicles stimulated by 1 and 0.5 microgram GTH / ml had significantly greater production of T at 1100h compared to 1900h. Intermediate and small follicles had significantly greater production of T at 1100h compared to 1900h throughout the whole range of GTH doses used. Both large and intermediate follicles produced similar T levels (100-150 pg T / mg protein) at the highest GTH dose.

Detectable levels of E_2 per mg of protein were produced by large, intermediate and small follicles when incubated with buffer alone; intermediate follicles without GTH stimulation produced significantly more E_2 at 1100h than at 1900h (Fig. 3). GTH caused a significant increase in the E_2 levels produced by all the three sizes of follicles. Large follicles produced more E_2 at 1100h than at 1900h under all dosages of GTH, except for the highest dose used (1 microgram GTH / ml). Intermediate follicles produced significantly greater levels of E_2 at 1100h compared to 1900h under all dosages of GTH, except for the lowest dose tested. For the small follicles, the E_2 levels produced were significantly greater at 1100h at only the two highest dosages of GTH. Relative to the large and intermediate follicles, small follicles showed the least E_2 production as assessed by steroid production per mg of protein. Intermediate follicles produced the highest E_2 levels, reaching 600 pg E_2 / mg protein at the highest dose of GTH tested.

Figure 2. Testosterone (T) production per milligram of protein by large, intermediate and small ovarian follicles under varying doses of carp gonadotropin (0.01-1.0 microgram cGTH/ml) or in buffer alone (Control) at two times (1100h and 1900h) during the day in January, 1983. Follicles were from goldfish acclimated for 5-7 days in 16L:8D/20°C in January, 1983. The gonadosomatic index (GSI) and the total number of fish killed (*n*) for the three consecutive days of sampling were pooled and the values presented are the mean±SEM of 10 to 15 replicates.

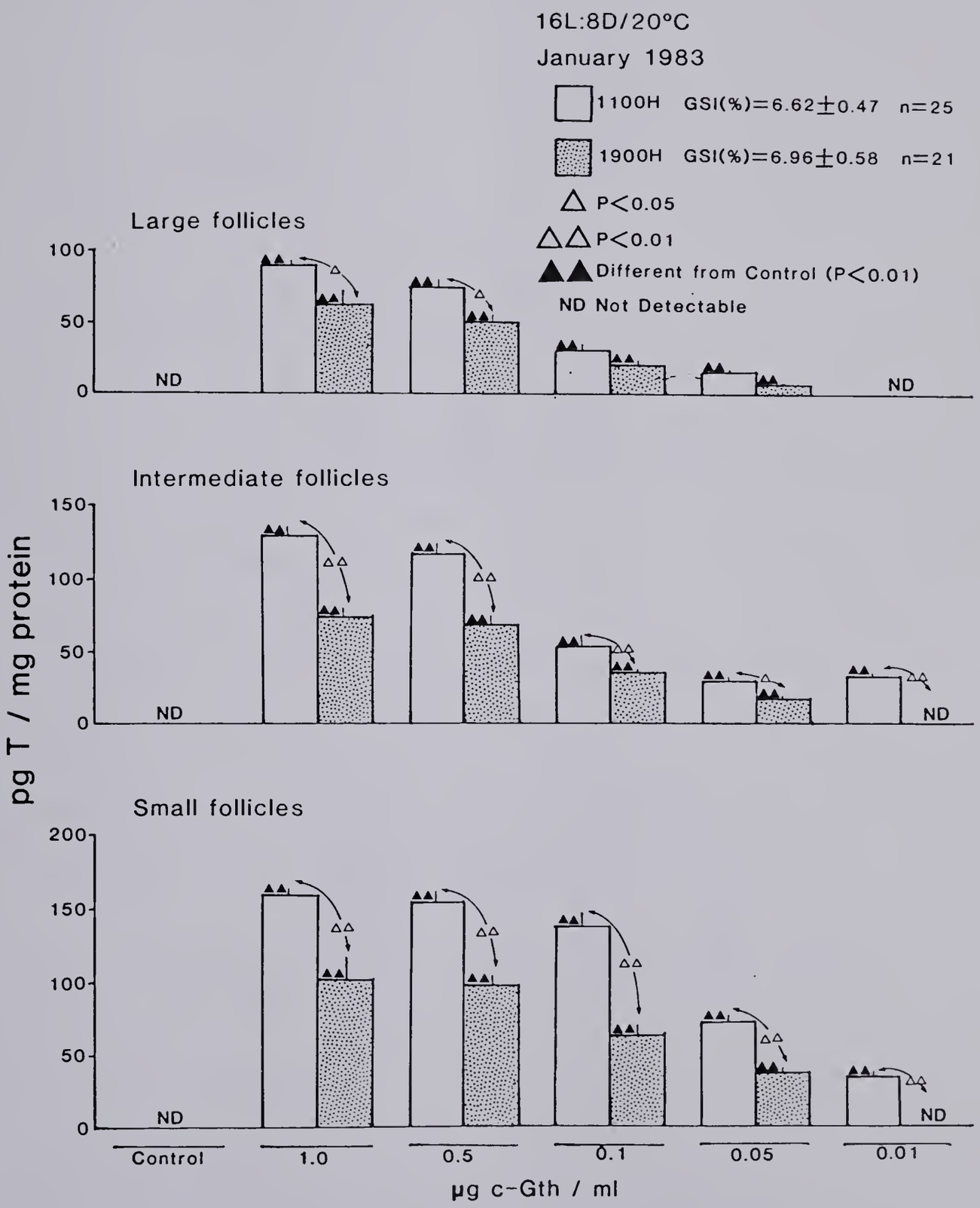
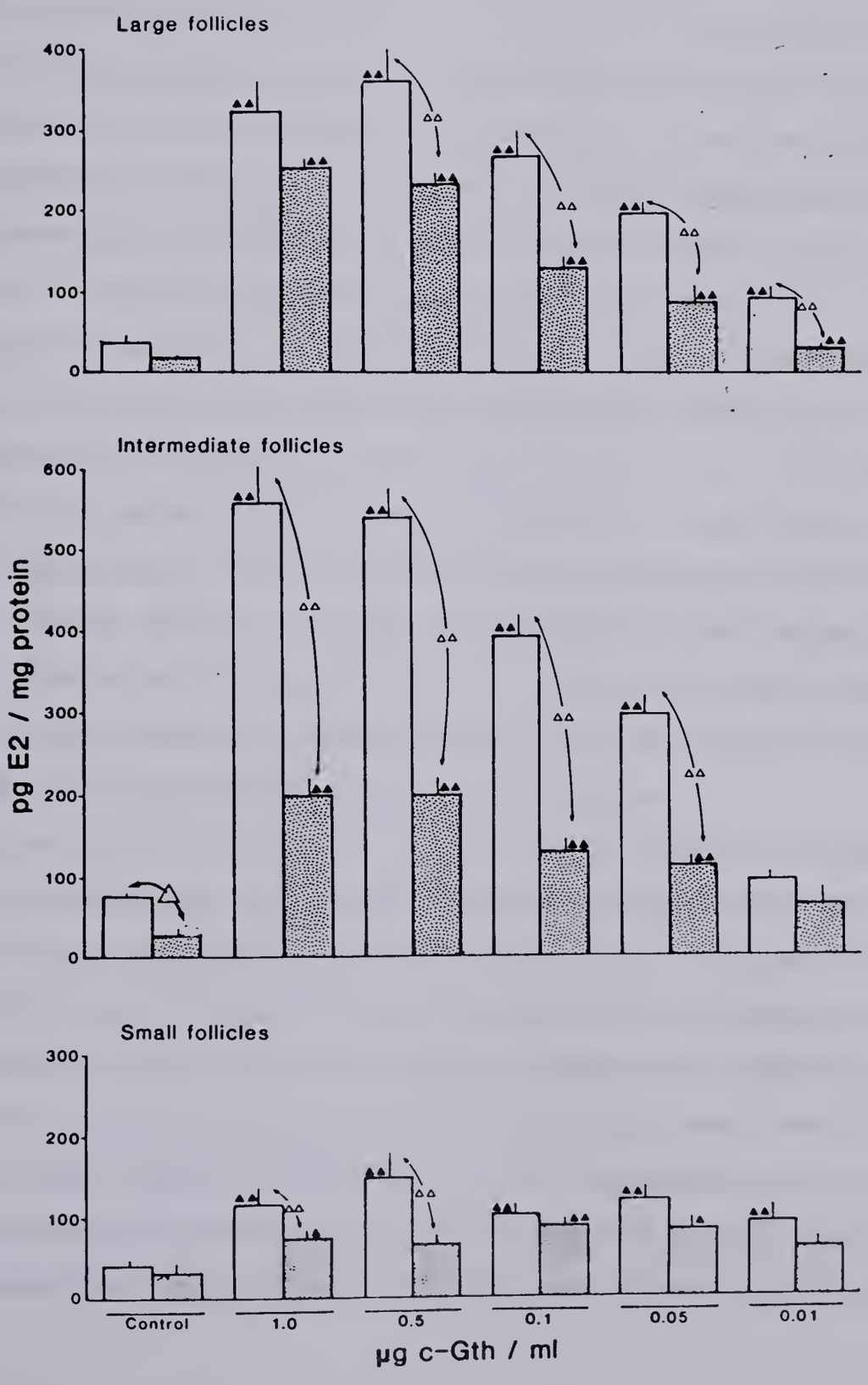

16L:8D/20°C
January 1983
1100H GSI(%)=6.62±0.47 n=25
1900H GSI(%)=6.96±0.58 n=21
△ P<0.05
△△ P<0.01
▲▲ Different from Control (P<0.01)
ND Not Detectable
Large follicles
Intermediate follicles
Small follicles
pg T / mg protein
ND
Control
1.0
0.5
0.1
0.05
0.01
µg c-Gth / ml

Figure 3. Estradiol-17 beta (E_2) production per milligram of protein by large, intermediate and small ovarian follicles under varying doses of carp gonadotropin (0.01-1.0 microgram cGTH/ml) or in buffer alone (Control) at two times (1100h and 1900h) during the day in January, 1983. Follicles were from goldfish acclimated for 5-7 days in 16L:8D/20°C in January, 1983. The gonadosomatic index (GSI) and the total number of fish killed (*n*) for the three consecutive sampling days are indicated. The results gathered from the three consecutive days of sampling were pooled and the values presented are the mean±SEM of 10 to 15 replicates.


16L:8D / 20°C
January 1983
1100H GSI(%)=6.62±0.47 n=25
1900H GSI(%)=6.96±0.58 n=21
△ P<0.05
△△ P<0.01
▲ Different from Control (P<0.05)
▲▲ Different from Control (P<0.01)
Large follicles
Intermediate follicles
Small follicles
pg E2 / mg protein
0
100
200
300
400
500
600
Control
1.0
0.5
0.1
0.05
0.01
µg c-Gth / ml

Testosterone production by all sizes of follicles was relatively constant at 100-150 pg T/mg protein at the highest dose of GTH used. However, E_2 production by large and intermediate follicles at this time of the year was 2-4 fold greater than small follicles and than T production by similar-sized follicles at the highest GTH dose.

2. April, 1983

The T and E_2 production per mg of protein by large and intermediate follicles sampled at two different times of the day from "spring" fish in April, 1983 after 5-7 days in 16L:8D/20°C subsequent to 8 days at 12L:12D/12°C are described in Figs. 4 and 5. There were insufficient small follicles to carry out incubations at this time of the year.

Large follicles incubated without GTH did not produce a detectable level of T whereas intermediate follicles did (Fig. 4). Incubation with GTH caused a significant stimulation of T production by large follicles. Incubation with higher dosages of GTH also caused a significant increase in T production per mg of protein by intermediate follicles at two times during the day. Large follicles from fish sampled at 1900h incubated with the lower dosages of GTH produced significantly higher T levels than at 1100h; however, no significant differences between time of day were detected for large follicles stimulated by the two highest doses of GTH. Intermediate-sized follicles showed no differences between 1100h and 1900h in their T production under GTH stimulation except at the lowest GTH dose tested. The magnitude of responsiveness of both follicle sizes was similar, reaching maximal values of approximately 100-150 pg T/mg protein at the highest GTH dose and 10-15 pg T/mg protein at the lowest GTH dose.

Incubation with most dosages of GTH at 1100h and 1900h caused a significant increase in E_2 production per mg of protein by large follicles from "spring" fish (Fig. 5). Incubation of intermediate follicles with GTH at the 0.1 and 0.01 microgram GTH/ml dose levels resulted in higher E_2 production levels compared with their untreated controls at 1100h; incubation with GTH at the three highest dose levels caused a significant stimulation of E_2 output by intermediate follicles compared to E_2 levels without GTH treatment at 1900h. Except for the lowest GTH dose, E_2 production per mg of protein by large follicles sampled at 1900h were significantly greater than at 1100h. However, with the intermediate-sized follicles, no significant differences in E_2 production under GTH

Figure 4. Testosterone (T) production per milligram of protein by large and intermediate ovarian follicles under varying doses of carp gonadotropin (0.01-1.0 microgram cGTH/ml) or in buffer alone (Control) at two times (1100h and 1900h) during the day in April, 1983. The gonadosomatic index (GSI) and the total number of fish killed (*n*) for the three consecutive sampling days are indicated. The results gathered from the three consecutive sampling days were pooled and the values presented are the mean±SEM of 10 to 15 replicates.

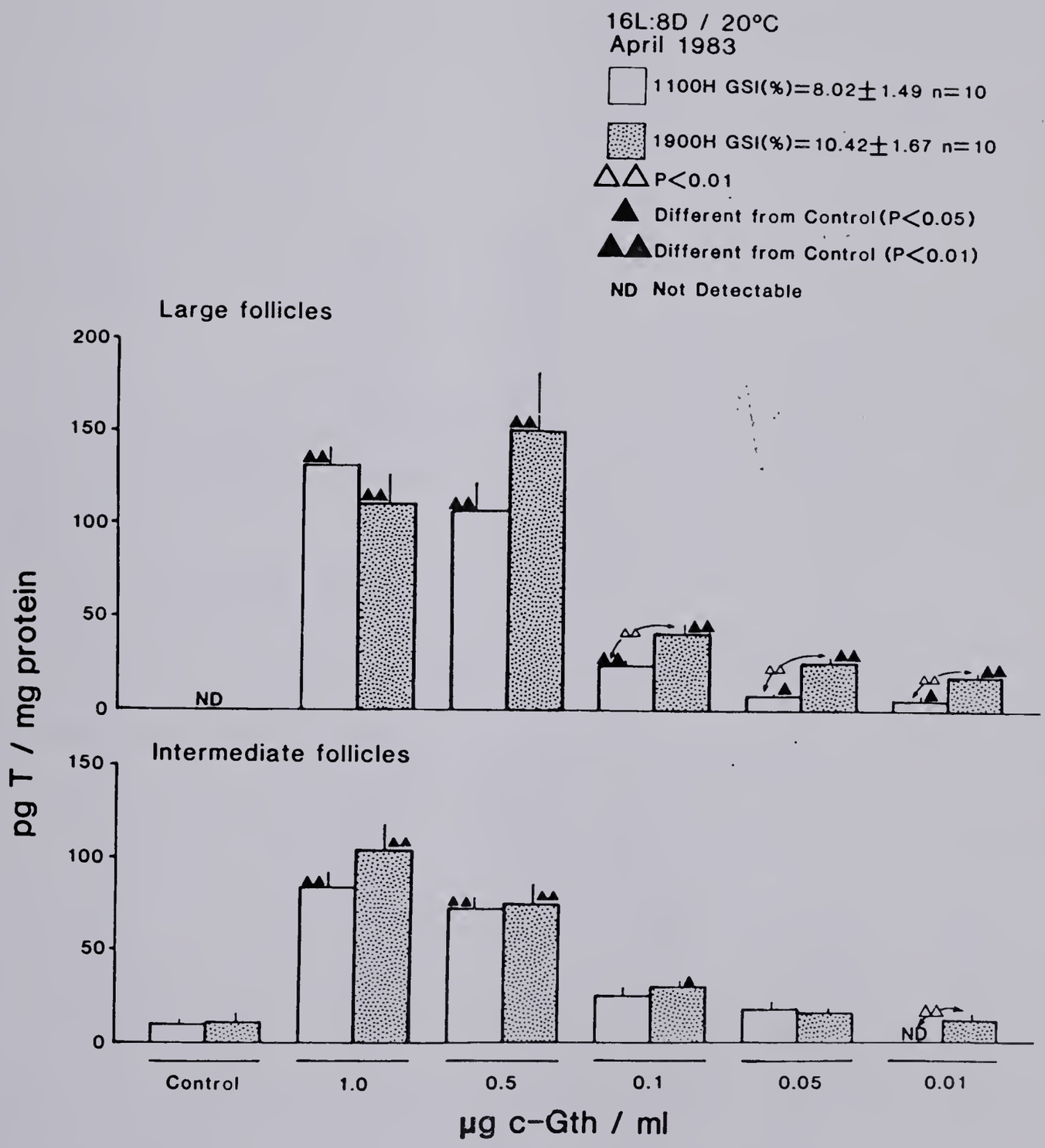
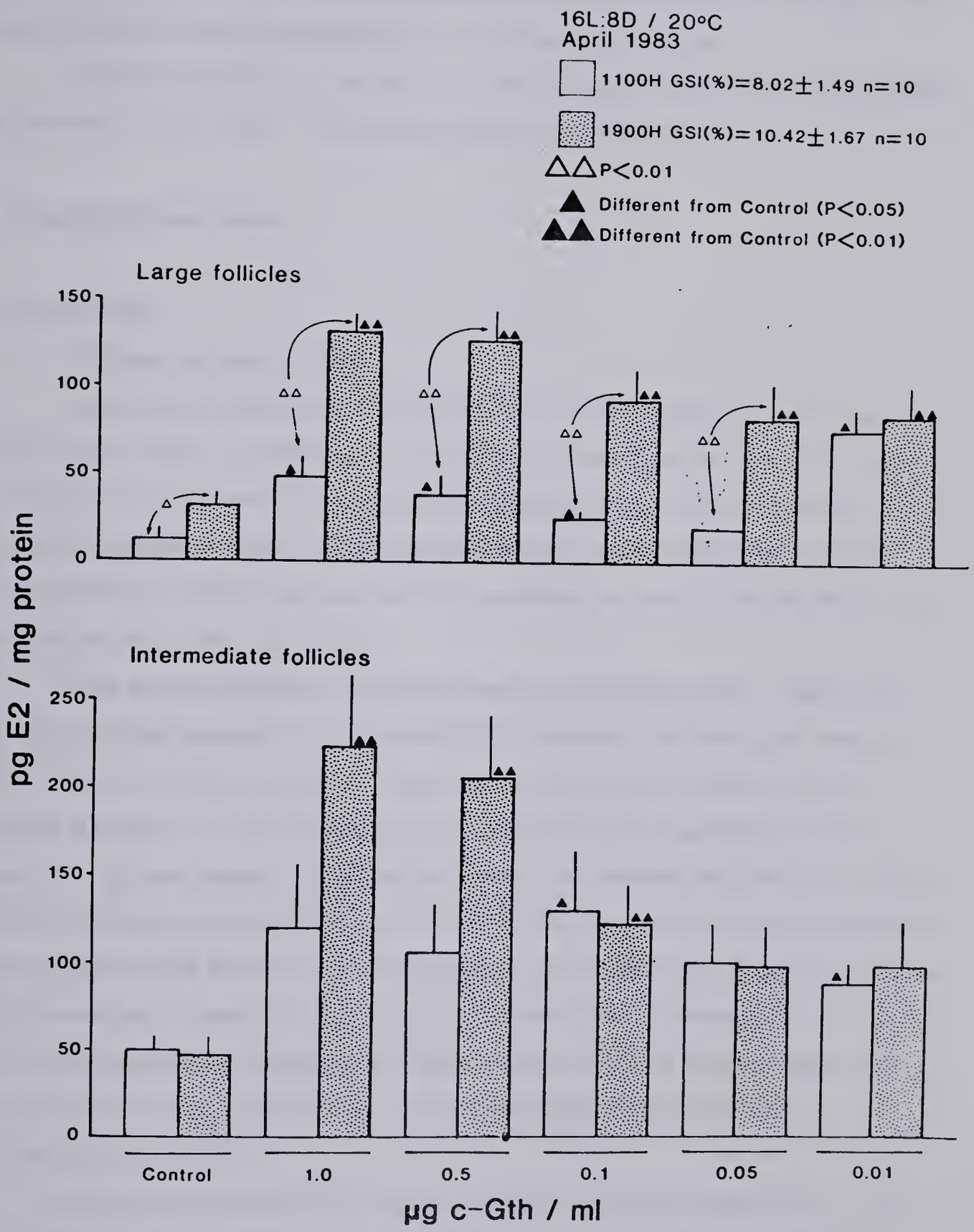

16L:8D / 20°C
April 1983
1100H GSI(%)=8.02±1.49 n=10
1900H GSI(%)=10.42±1.67 n=10
P<0.01
Different from Control(P<0.05)
Different from Control (P<0.01)
ND Not Detectable
Large follicles
Intermediate follicles
pg T / mg protein
200
150
100
50
0
ND
Control
1.0
0.5
0.1
0.05
0.01
µg c-Gth / ml

Figure 5. Estradiol-17 beta (E_2) per milligram of protein by large and intermediate ovarian follicles under varying doses of carp gonadotropin (0.01-1.0 microgram cGTH/ml) or in buffer alone (Control) at two times (1100h and 1900h) during the day in April, 1983. Follicles were from goldfish acclimated for 5-7 days in 16L:8D/20°C in April, 1983. The gonadosomatic index (GSI) and the total number of fish killed (*n*) for the three consecutive sampling days were pooled and the values presented are the mean±SEM of 10 to 15 replicates.


16L:8D / 20°C
April 1983
1100H GSI(%)=8.02±1.49 n=10
1900H GSI(%)=10.42±1.67 n=10
△△P<0.01
▲ Different from Control (P<0.05)
▲▲ Different from Control (P<0.01)
Large follicles
Intermediate follicles
pg E2 / mg protein
150
100
50
0
250
200
150
100
50
0
Control
1.0
0.5
0.1
0.05
0.01
µg c-Gth / ml

stimulation were detected between the two different times of day. The E_2 production of both follicle sizes was similar, except for the intermediate follicles stimulated by the two highest doses where the E_2 levels produced were statistically higher (200-250 pg E_2/mg protein) than their respective counterparts in the large-sized follicles.

Testosterone and E_2 production at this time of the year were generally comparable, with maximal values close to 150 and 200 pg steroid/mg of protein.

B. Long-Term Experiments

1. 16L:8D/20°C

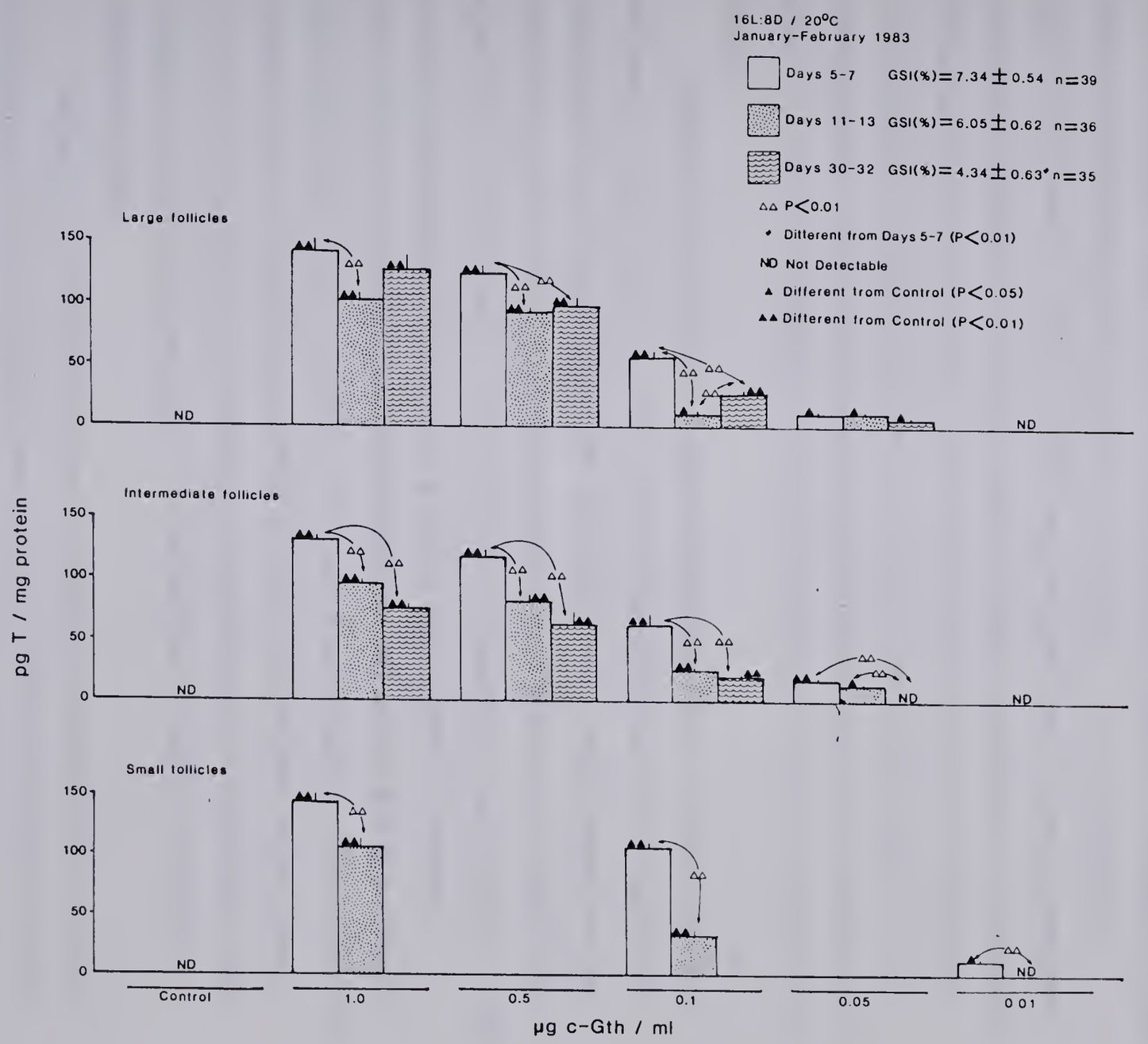
a. January-February, 1983

Follicles were sampled at two different times of day at Days 5-7 , 11-13 and 30-32 from "winter" fish acclimated to 16L:8D/20°C, subsequent to an initial 8 days at 12L:12D/ 12°C. The mean GSI at Days 30-32 was significantly lower than at Days 5-7. Due to the small sample sizes of some of the individual sets of samples and variability within the individual sets of samples, all of the replicates over each three day period were grouped and mean values expressed.

There was no detectable T production per mg of protein by large , intermediate and small follicles incubated in buffer alone (Fig. 6). Incubation with the higher dosages of GTH caused a significant increase in T levels per mg of protein by all three sizes of follicles at Days 5-7, 11-13 and 30-32. Increasing the period of acclimation from 5-7 days to 11-13 days caused a significant decrease in the T production per mg of protein of large, intermediate and small follicles in the 0.1-1.0 microgram GTH/ml range. Large and intermediate follicles taken from fish acclimated to 16L:8D/20°C for 30-32 days also had significantly less T production per mg of protein under GTH stimulation than was found at 5-7 days of acclimation, except for the highest dose and the two lower dosages of GTH with the large follicles. There was not an adequate number of small follicles for incubations at Days 30-32.

The E_2 production per mg of protein by all sizes of follicles at Days 5-7, 11-13 and 30-32 were stimulated by most dosages of GTH compared to their respective untreated control groups. The E_2 production per mg of protein by large follicles in

Figure 6. Testosterone (T) production per milligram of protein by large, intermediate and small ovarian follicles under varying doses of carp gonadotropin (0.01-1.0 microgram cGTH/ml) or in buffer alone (Control) after goldfish were acclimated for 5-7, 11-13 and 30-32 days in 16L:8D/20°C in January-February, 1983. The gonadosomatic index (GSI) and the total number of fish killed (*n*) for the three consecutive sampling days are indicated. Ovarian follicles were sampled at different times during the day and each value, expressed as the mean±SEM of 10 to 30 replicates, represents data pooled from the three consecutive sampling days as outlined in Fig. 1.


16L:8D / 20°C
January-February 1983
Days 5-7 GSI(%)=7.34±0.54 n=39
Days 11-13 GSI(%)=6.05±0.62 n=36
Days 30-32 GSI(%)=4.34±0.63* n=35
△△ P<0.01
* Different from Days 5-7 (P<0.01)
ND Not Detectable
▲ Different from Control (P<0.05)
▲▲ Different from Control (P<0.01)
Large follicles
Intermediate follicles
Small follicles
pg T / mg protein
150
100
50
0
ND
Control
1.0
0.5
0.1
0.05
0 01
µg c-Gth / ml

response to high dosages of GTH showed an abrupt decrease between Days 5-7 and Days 11-13 of acclimation to 16L:8D / 20°C (Fig. 7). The E_2 levels per mg of protein at Days 30-32 were also significantly lower than at Days 5-7 except at the two lowest GTH doses where levels were similar to Days 5-7. E_2 production per mg of protein by large follicles at Days 11-13 and 30-32 were similar in controls and at all GTH doses with the exception of the lowest dose. Likewise, the E_2 production per mg of protein by intermediate follicles exhibited an abrupt decrease between Days 5-7 and Days 11-13 of acclimation to 16L:8D / 20°C; however, at the lowest GTH dose, no significant differences were noted with length of acclimation. Interestingly, after 30-32 days of acclimation, the intermediate follicles had similar responsiveness as intermediate follicles at Days 5-7 and generally produced significantly greater amounts of E_2 per mg of protein than at Days 11-13. For the small follicles, the E_2 levels produced per mg of protein also showed a significant drop at Days 11-13 compared to Days 5-7, except for the controls and at the lowest GTH dose tested.

The results in Figs. 6 and 7 were also transformed, in part, to mean values of E_2 and T per unit surface area (mm^2) of the follicles at the highest dose of GTH tested and presented in Fig. 8. The E_2 production per unit surface area of large follicles from fish sampled at Days 5-7 was two fold greater than by large follicles at other sample times during the experimental period. Large follicles also consistently produced greater E_2 levels per unit surface area compared to the other follicle sizes. Under GTH stimulation, intermediate and small follicles had similar E_2 levels per unit surface area throughout the 32-day experimental period. The T production per unit surface area by GTH-treated large follicles at Days 5-7 and 30-32 were similar but both were less than T levels at Days 11-13. The T production per unit surface area by large follicles was significantly higher compared to other follicle sizes at any of the sample times throughout the 32-day experimental period.

The results presented in Figs. 6 and 7 were also transformed, in part, to E_2 and T production per follicle at the highest dose of GTH tested and are presented in Fig. 9. E_2 production per follicle by GTH-treated large follicles was highest after 5-7 days at 16L:8D / 20°C but E_2 levels after 11-13 and 30-32 days in this environmental regime were similar and lower than at Days 5-7. Under GTH stimulation, large follicles had higher E_2

Figure 7. Estradiol-17 beta (E_2) production per milligram of protein by large, intermediate and small ovarian follicles under varying doses of carp gonadotropin (0.01-1.0 microgram cGTH/ml) or in buffer alone (Control) after goldfish were acclimated for 5-7, 11-13 and 30-32 days in 16L:8D/20°C in January-February, 1983. The gonadosomatic index (GSI) and the total number of fish killed (*n*) for the three consecutive sampling days are indicated. Ovarian follicles were sampled at different times during the day and each value, expressed as the mean±SEM of 10 to 30 replicates, represents data pooled from the three consecutive sampling days as outlined in Fig. 1.

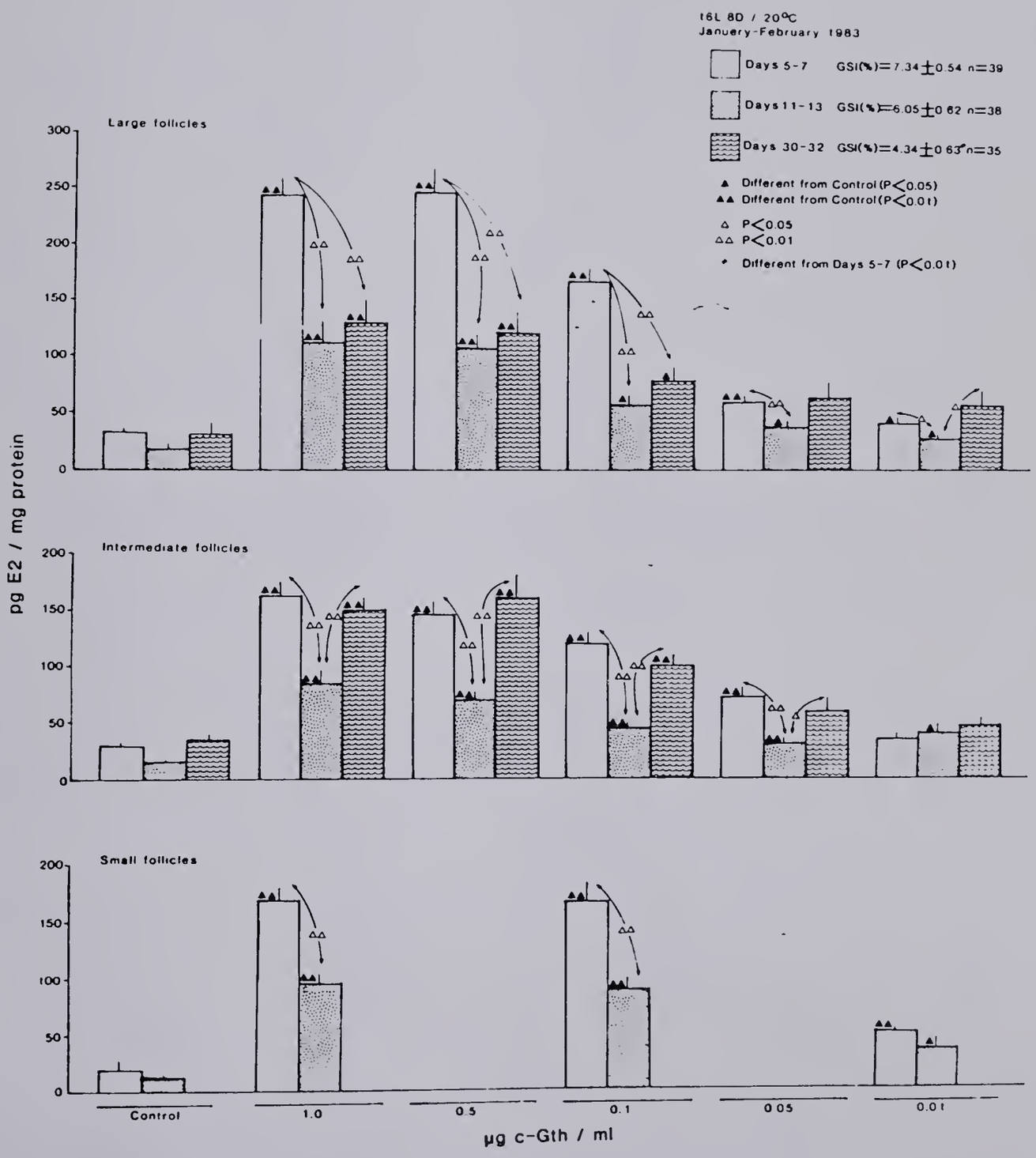

16L 8D / 20°C
January-February 1983
Days 5-7 GSI(%)=7.34±0.54 n=39
Days 11-13 GSI(%)=6.05±0.62 n=38
Days 30-32 GSI(%)=4.34±0.63 n=35
▲ Different from Control (P<0.05)
▲▲ Different from Control (P<0.01)
△ P<0.05
△△ P<0.01
• Different from Days 5-7 (P<0.01)
Large follicles
Intermediate follicles
Small follicles
pg E2 / mg protein
300
250
200
150
100
50
0
Control
1.0
0.5
0.1
0.05
0.01
µg c-Gth / ml

Figure 8. Estradiol-17 beta (E_2) and testosterone (T) production per unit surface area (mm^2) by carp gonadotropin-treated (1 microgram cGTH/ml) and untreated (Control) large, intermediate and small ovarian follicles from goldfish acclimated for 5-7, 11-13 and 30-32 days in 16L:8D/20°C in January-February, 1983. Each value, expressed as the mean±SEM of 10 to 30 replicates, was transformed from data presented in Figs. 6 and 7. The results of a Duncan's multiple range test ($P<0.05$) are indicated; means which are not significantly different from each other are underlined.

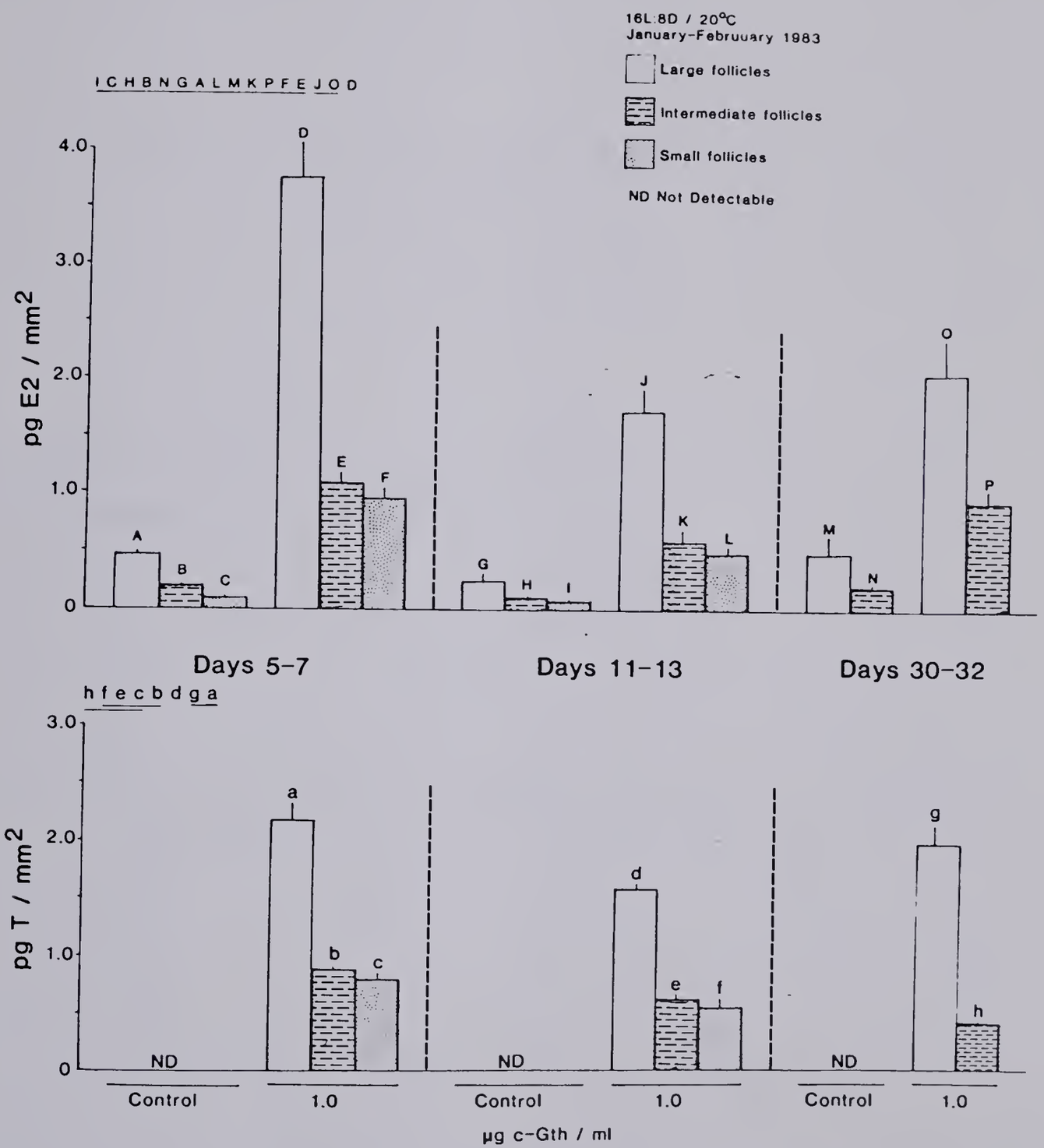
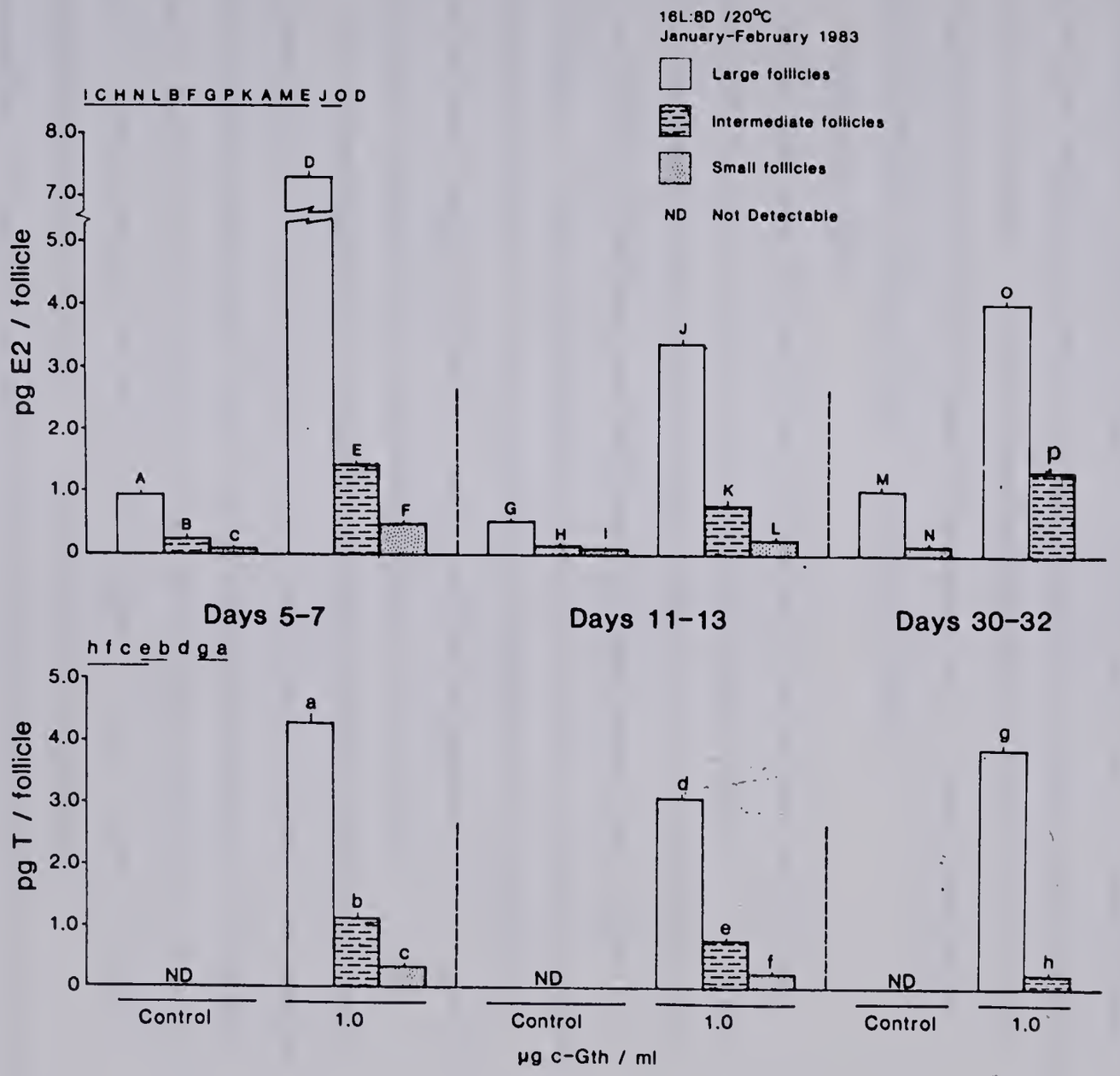

16L:8D / 20°C
January-February 1983
Large follicles
Intermediate follicles
Small follicles
ND Not Detectable
I C H B N G A L M K P F E J O D
pg E2 / mm2
4.0
3.0
2.0
1.0
0
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Days 5-7
Days 11-13
Days 30-32
h f e c b d g a
pg T / mm2
3.0
2.0
1.0
0
a
b
c
d
e
f
g
h
ND
ND
ND
Control
1.0
Control
1.0
Control
1.0
µg c-Gth / ml

Figure 9. Estradiol-17 beta (E_2) and testosterone (T) production per follicle by carp gonadotropin-treated (1 microgram cGTH/ml) and untreated (Control) large, intermediate and small ovarian follicles from goldfish acclimated for 5-7, 11-13 and 30-32 days in 16L:8D/20°C in January-February, 1983. Each value, expressed as the mean±SEM of 10 to 30 replicates, was transformed from data presented in Figs. 6 and 7. The results of a Duncan's multiple range test ($P<0.05$) are indicated; means which are not significantly different from each other are underlined.


16L:8D /20°C
January-February 1983
Large follicles
Intermediate follicles
Small follicles
ND Not Detectable
I C H N L B F G P K A M E J O D
pg E2 / follicle
8.0
7.0
5.0
4.0
3.0
2.0
1.0
0
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Days 5-7
Days 11-13
Days 30-32
h f c e b d g a
pg T / follicle
5.0
4.0
3.0
2.0
1.0
0
a
b
c
d
e
f
g
h
ND
ND
ND
Control
1.0
Control
1.0
Control
1.0
µg c-Gth / ml

production per follicle than any of the other follicle sizes throughout the 32-day experimental period. Intermediate and small follicles under GTH stimulation had similar E_2 output per follicle throughout the 32-day experimental period. The T production per follicle by large follicles exposed to GTH was similar after 5-7 and 30-32 days in 16L:8D / 20°C; however, T production was significantly lower at Days 11-13 compared to Days 5-7 and 30-32. Under GTH stimulation, T production per follicle by large follicles was consistently more elevated compared to the other follicle sizes at all sample times. Levels of T per follicle by intermediate follicles under GTH stimulation were similar at Days 5-7 and 11-13; however, T production by GTH-treated small follicles at Days 5-7 and 11-13 and intermediate follicles at Days 30-32 were similar to T levels produced per follicle by intermediate follicles at Days 11-13 but not at Days 5-7.

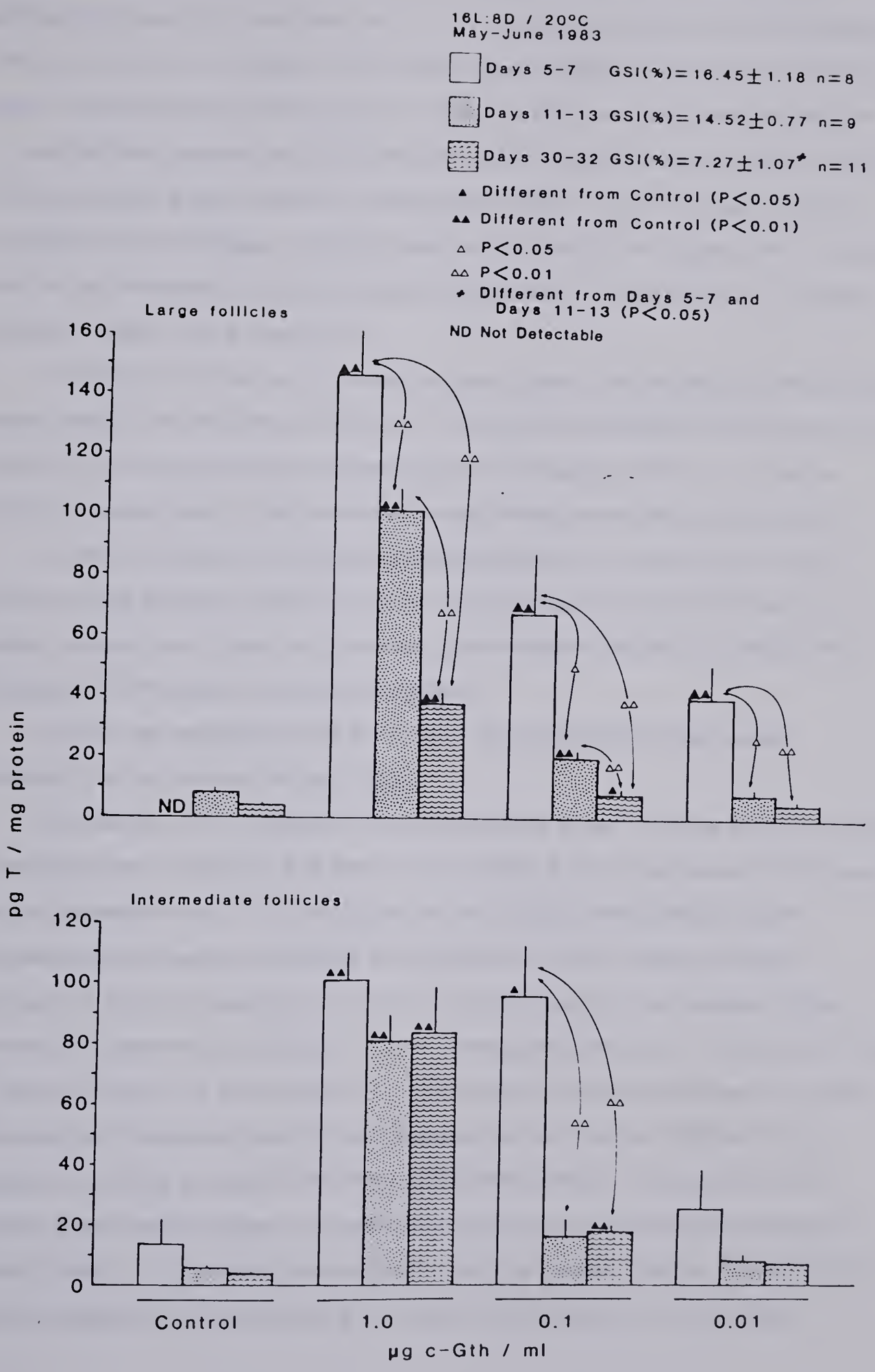
b. May-June, 1983

GTH at the 1 and 0.01 microgram GTH / ml dose levels caused a significant stimulation of T production per mg of protein by large follicles at Days 5-7, 11-13 and 30-32. T production per mg of protein by large follicles taken from fish sampled at Days 5-7 after 16L:8D / 20°C in May-June, 1983 was significantly greater than at Days 11-13 and 30-32 (Fig. 10). There was a progressive decrease in T levels produced per milligram of protein from Days 5-7, to 11-13, to 30-32 under stimulation with high dosages of GTH; at the lowest dosage of GTH (0.01 microgram GTH / ml) used there was no significant stimulation of T production over control levels at Days 11-13 and 30-32.

At most dosages of GTH, T production per mg of protein by intermediate follicles were greater than their controls throughout the 32-day experimental period. Intermediate follicles produced similar levels of T per mg of protein under stimulation of GTH at either 1.0 and 0.01 microgram GTH / ml throughout the 32-day experimental period. However, at a dose of 0.1 microgram GTH / ml, the T levels produced per mg of protein at Days 5-7 were significantly higher than at Days 11-13 and 30-32. The magnitude of T responsiveness of large and intermediate follicles was similar, attaining levels of 100-150 pg T / mg protein under stimulation with the highest dose of GTH (1.0 microgram GTH / ml) at Days 5-7.

Under all GTH dosages tested, E_2 production per mg of protein by large and intermediate follicles at Days 5-7 and 11-13 was greater compared to their untreated

Figure 10. Testosterone (T) production per milligram of protein by large and intermediate ovarian follicles under varying doses of carp gonadotropin (0.01-1.0 microgram cGTH/ml) or in buffer alone (Control) after goldfish were acclimated for 5-7, 11-13 and 30-32 days in 16L:8D/20°C in May-June, 1983. The gonadosomatic index (GSI) and the total number of fish killed (*n*) for the three consecutive sampling days are indicated. Ovarian follicles were sampled at different times during the day and each value, expressed as the mean±SEM of 15 to 30 replicates, represents data pooled from the three consecutive sampling days as outlined in Fig. 1.


16L:8D / 20°C
May-June 1983
Days 5-7 GSI(%)=16.45±1.18 n=8
Days 11-13 GSI(%)=14.52±0.77 n=9
Days 30-32 GSI(%)=7.27±1.07* n=11
▲ Different from Control (P<0.05)
▲▲ Different from Control (P<0.01)
△ P<0.05
△△ P<0.01
* Different from Days 5-7 and Days 11-13 (P<0.05)
ND Not Detectable
Large follicles
Intermediate follicles
pg T / mg protein
ND
0
20
40
60
80
100
120
140
160
Control
1.0
0.1
0.01
µg c-Gth / ml

control group (Fig. 11). Notably, at Days 30-32, GTH was effective in stimulating a significant increase in E_2 production per mg of protein by large follicles over its untreated control at only the 0.5 microgram GTH/ml dose level. E_2 production per mg of protein by large follicles taken from fish at Days 5-7 at 16L:8D/20°C was significantly higher than for large follicles taken at Days 30-32; however, the E_2 levels produced by large follicles were significantly greater at Days 5-7 compared to Days 11-13 at only the 0.5 and 0.1 microgram GTH/ml dosages. At all GTH doses, except for 0.5 microgram GTH/ml, the E_2 level per mg of protein produced by large follicles taken from fish at Days 11-13 were significantly higher than at Days 30-32.

The levels of E_2 per mg of protein produced under GTH stimulation by intermediate follicles taken from fish sampled at Days 5-7 were significantly higher than at Days 30-32. Levels of E_2 per mg of protein produced under GTH stimulation at Days 11-13 were similar to those at Days 5-7 but were always significantly greater than at Days 30-32.

Under stimulation by the highest GTH concentration, E_2 production per mg of protein by large follicles at Days 5-7 and 11-13 was at approximately 20-50 pg/mg protein; production of E_2 per mg of protein by intermediate follicles stimulated by this same dose of GTH was two to three fold greater.

GSI of fish sampled on Days 5-7 and 11-13 were similar and decreased significantly in fish sampled on Days 30-32.

The results in Figs. 10 and 11 were transformed, in part, to mean values of E_2 and T production per unit surface area (mm^2) of the follicles at the highest dose of GTH tested and are presented in Fig. 12. E_2 production per unit surface area by large follicles progressively decreased as the length of acclimation to 16L:8D/ 20°C increased. However, E_2 levels produced per unit surface area produced by intermediate follicles were at the highest values at Days 11-13, and were significantly lower at Days 30-32 than at Days 5-7 and 11-13. Only at Days 11-13 were the E_2 levels produced per unit surface area by large follicles significantly lower compared to intermediate follicles. Control groups were similar throughout the entire experimental period. T production per unit surface area by large follicles was significantly greater than by intermediate follicles at Days 5-7 and 11-13, but decreased to the level of intermediate follicles at Days 30-32. T production per unit surface area by GTH-treated large follicles also progressively

Figure 11. Estradiol-17 beta (E_2) production per milligram of protein by large and intermediate ovarian follicles under varying doses of carp gonadotropin (0.01-1.0 microgram cGTH/ml) or in buffer alone (Control) after goldfish were acclimated for 5-7, 11-13 and 30-32 days in 16L:8D/20°C in May-June, 1983. The gonadosomatic index (GSI) and the total number of fish killed (*n*) for the three consecutive sampling days are indicated. Ovarian follicles were sampled at different times during the day and each value, expressed as the mean±SEM of 10 to 30 replicates, represents data pooled from the three consecutive sampling days as outlined in Fig. 1.

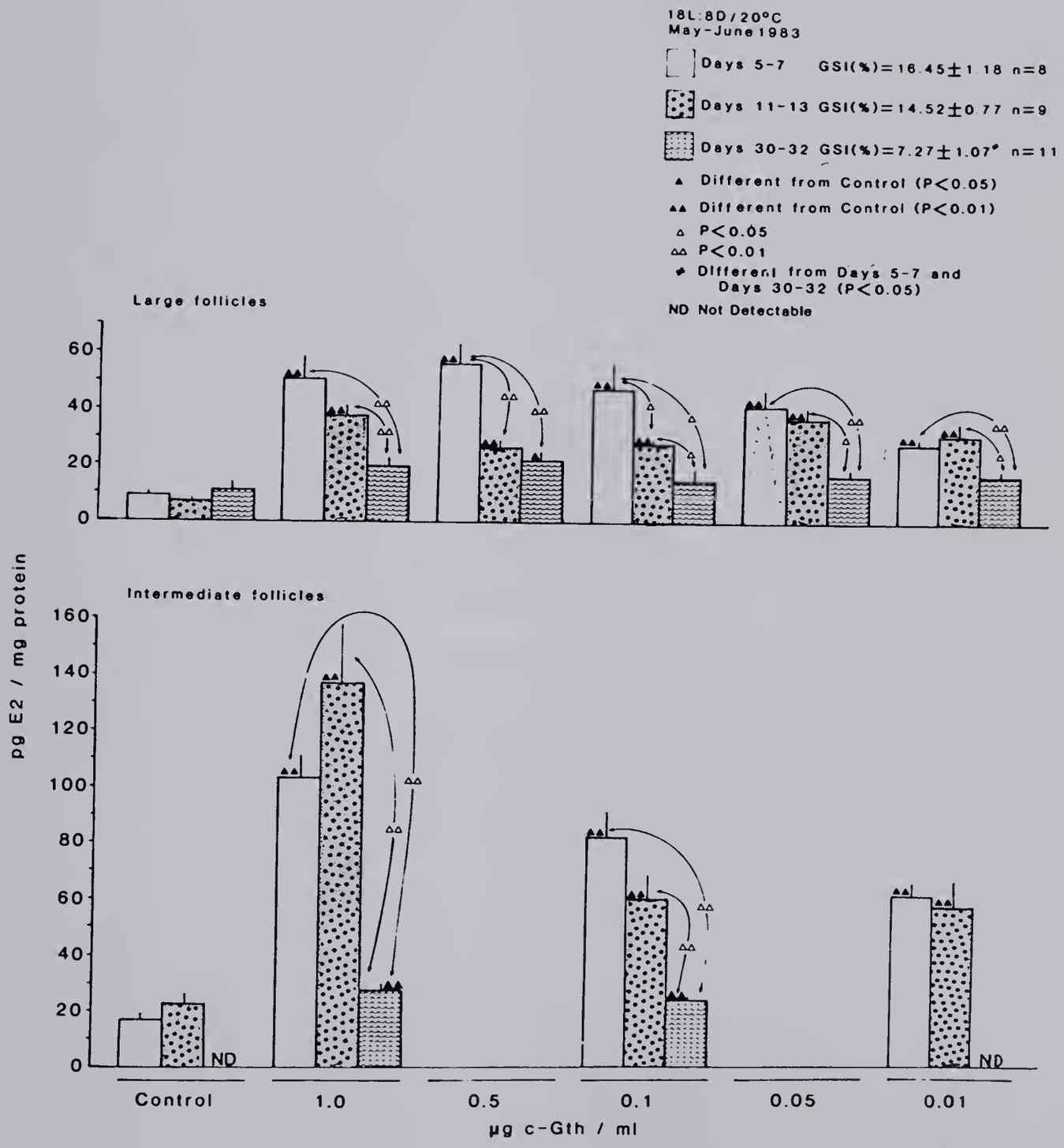
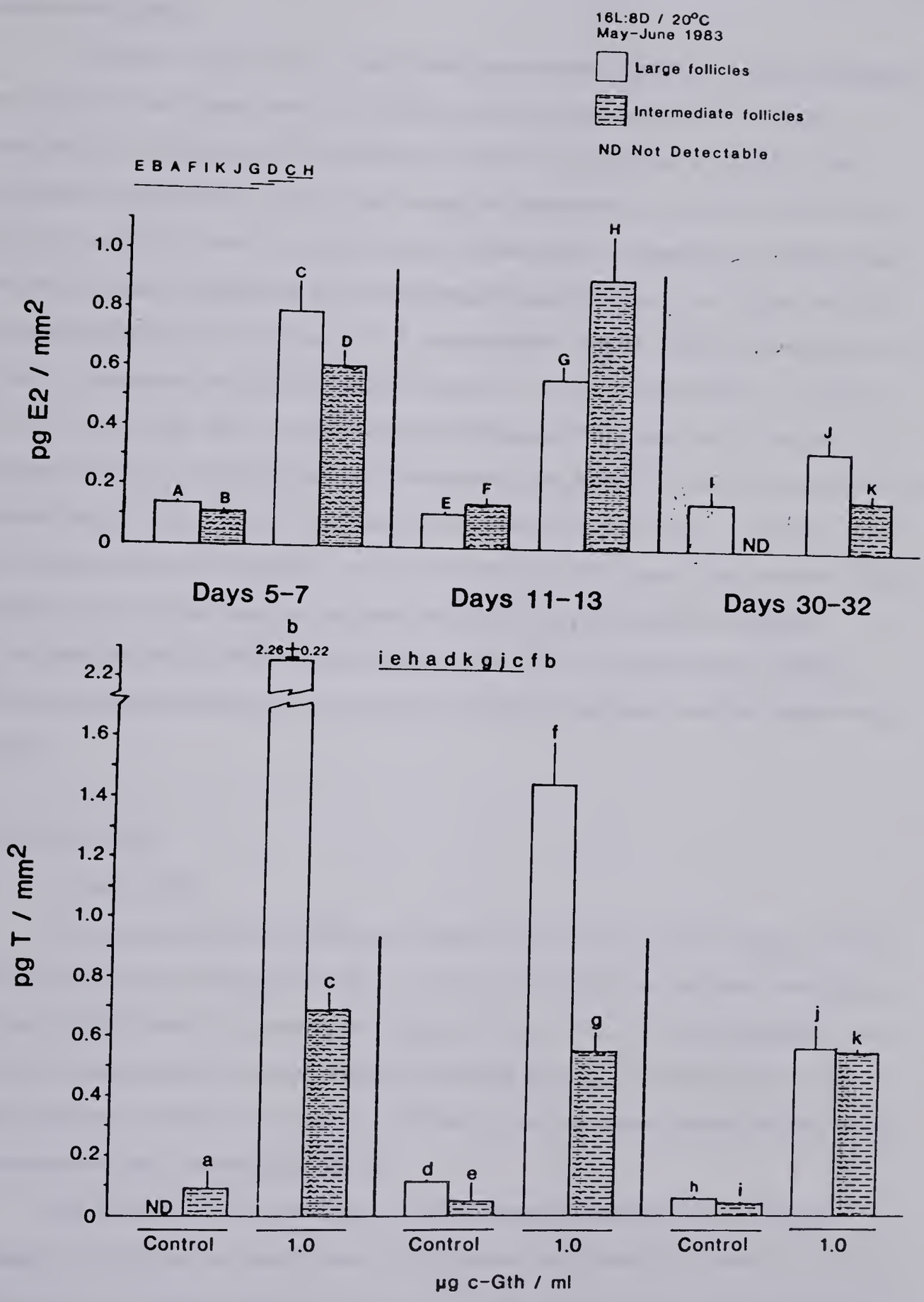

18L:8D/20°C
May-June 1983
Days 5-7 GSI(%)=16.45±1.18 n=8
Days 11-13 GSI(%)=14.52±0.77 n=9
Days 30-32 GSI(%)=7.27±1.07* n=11
▲ Different from Control (P<0.05)
▲▲ Different from Control (P<0.01)
△ P<0.05
△△ P<0.01
* Different from Days 5-7 and Days 30-32 (P<0.05)
ND Not Detectable
Large follicles
Intermediate follicles
pg E2 / mg protein
ND
ND
Control
1.0
0.5
0.1
0.05
0.01
µg c-Gth / ml

Figure 12. Estradiol-17 beta (E_2) and testosterone (T) production per unit surface area (mm^2) by carp gonadotropin-treated (1 microgram cGTH/ml) and untreated (Control) large and intermediate ovarian follicles from goldfish acclimated for 5-7, 11-13 and 30-32 days in 16L:8D/20°C in May-June, 1983. Each value, expressed as the mean±SEM of 10 to 30 replicates, was transformed from data presented in Figs. 10 and 11. The results of a Duncan's multiple range test ($P<0.05$) are indicated; means which are not significantly different from each other are underlined.


16L:8D / 20°C
May-June 1983
Large follicles
Intermediate follicles
ND Not Detectable
E B A F I K J G D C H
pg E2 / mm2
1.0
0.8
0.6
0.4
0.2
0
A
B
C
D
E
F
G
H
I
ND
J
K
Days 5-7
Days 11-13
Days 30-32
b
2.26±0.22
i e h a d k g j c f b
pg T / mm2
2.2
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ND
a
b
c
d
e
f
g
h
i
j
k
Control
1.0
Control
1.0
Control
1.0
µg c-Gth / ml

decreased from Days 5-7 to 11-13 and to Days 30-32. T production per unit surface area by intermediate follicles remained constant under GTH stimulation throughout the 32-day experimental period.

The data in Figs. 10 and 11 were also transformed, in part, to T and E_2 production per follicle at the highest dose of GTH used, and are presented in Fig. 13. The E_2 production per follicle by GTH-treated large follicles was greatest at Days 5-7, and decreased progressively with increase in length of acclimation to 16L:8D / 20°C. Only at Days 5-7 and 30-32 was E_2 production per follicle by GTH-treated large follicles greater than the E_2 levels produced by GTH-treated intermediate follicles. Also, after reaching maximal production of E_2 at Days 11-13, intermediate follicles under GTH stimulation had similar E_2 production levels per follicle compared to large follicles at Days 11-13 and 30-32. On the other hand, T production per follicle by GTH-treated large follicles was greatest at Days 5-7 and progressively decreased with length of acclimation period. The T production per follicle by GTH-treated large follicles at Days 5-7 and 11-13 was significantly greater compared to T levels produced by GTH-treated intermediate follicles at these same sample times; only at Days 30-32 was T production per follicle by GTH-treated large and intermediate follicles similar. The T production per follicle by GTH-treated intermediate follicles remained constant throughout the entire experimental period.

2. 12L:12D / 12°C

a. March, 1983

Following an initial environmental regime of 12L:12D / 12°C for 8 days, the fish were subjected to continuation of the 12L:12D / 12°C regime and sampled during Days 6-8 and 30-32 (refer to Experimental Protocol in Fig. 1). The fish were sampled at two different times of day over each three day sampling period, follicles incubated with varying doses of cGTH for 4 hours at 12°C, and pooled values expressed as the steroid production at Days 6-8 and Days 30-32.

The production of T per mg of protein by large follicles at Days 6-8 in the presence of different concentrations of GTH was not significantly different from the T production by follicles incubated without GTH, except for the highest dose of GTH (Fig.

Figure 13. Estradiol-17 beta (E_2) and testosterone (T) production per follicle by carp gonadotropin-treated (1 microgram cGTH/ml) and untreated (Control) large and intermediate ovarian follicles from goldfish acclimated for 5-7, 11-13 and 30-32 days in 16L:8D/20°C in May-June, 1983. Each value, expressed as the mean±SEM of 10 to 30 replicates, was transformed from data presented in Figs. 10 and 11. The results of a Duncan's multiple range test ($P<0.05$) are indicated; means which are not significantly different from each other are underlined.

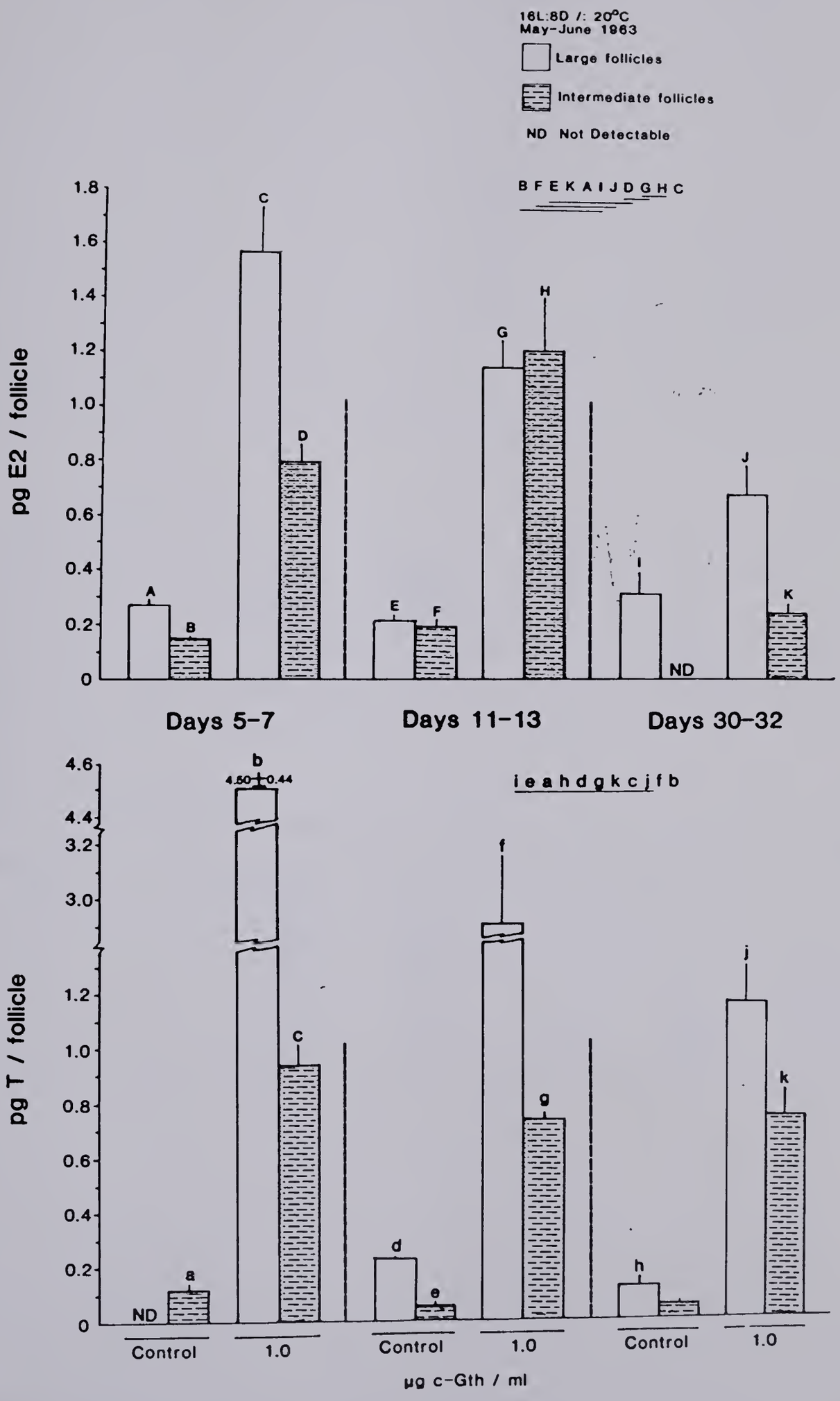
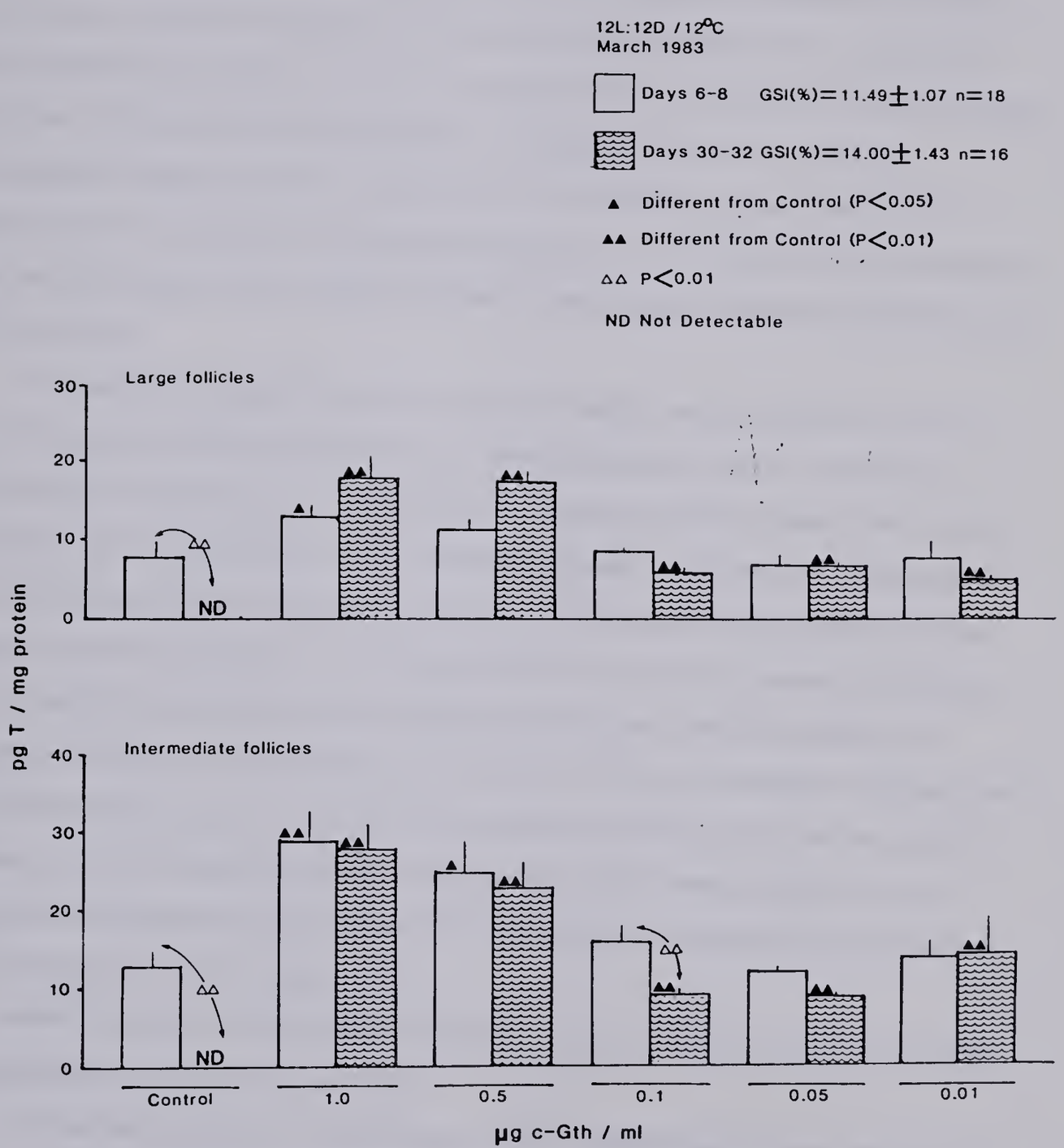

16L:8D /: 20°C
May-June 1983
Large follicles
Intermediate follicles
ND Not Detectable
B F E K A I J D G H C
pg E2 / follicle
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
A
B
C
D
E
F
G
H
I
ND
J
K
Days 5-7
Days 11-13
Days 30-32
i e a h d g k c j f b
pg T / follicle
4.6
4.4
3.2
3.0
1.2
1.0
0.8
0.6
0.4
0.2
0
b
4.50 ± 0.44
a
ND
c
d
e
f
g
h
j
k
Control
1.0
Control
1.0
Control
1.0
µg c-Gth / ml

Figure 14. Testosterone (T) production per milligram of protein by large and intermediate ovarian follicles under varying doses of carp gonadotropin (0.01-1.0 microgram cGTH/ml) or in buffer alone (Control) after goldfish were acclimated for 6-8 and 30-32 days in 12L:12D/12°C in March, 1983. The gonadosomatic index (GSI) and the total number of fish killed (*n*) for the three consecutive sampling days are indicated. Ovarian follicles were sampled at different times during the day and each value, expressed as the mean±SEM of 10 to 25 replicates, represents data pooled from results gathered during the three consecutive sampling days as outlined in Fig. 1.


12L:12D / 12°C
March 1983
Days 6-8 GSI(%)=11.49±1.07 n=18
Days 30-32 GSI(%)=14.00±1.43 n=16
▲ Different from Control (P<0.05)
▲▲ Different from Control (P<0.01)
△△ P<0.01
ND Not Detectable
Large follicles
Intermediate follicles
pg T / mg protein
ND
Control
1.0
0.5
0.1
0.05
0.01
µg c-Gth / ml

14). GTH stimulated T production per mg of protein by large follicles at Days 30-32 over their untreated controls which had no detectable levels of T measured. Except for the untreated control groups, no significant differences were noted in T levels per mg of protein produced by large follicles exposed to similar concentrations of GTH between Days 6-8 and Days 30-32. The T production per mg of protein by intermediate follicles was also low; however, at Days 6-8 T production by intermediate follicles exposed to high concentrations of GTH (1.0 and 0.5 microgram GTH/ml) was significantly greater than in controls. At Days 30-32, GTH stimulated T production per mg of protein by intermediate follicles over their untreated controls which had no detectable level of T measured. Only in the control groups and at a dose of 0.1 microgram GTH/ml was there a significant difference in the T levels produced by intermediate follicles between Days 6-8 and Days 30-32.

Although E_2 production per mg of protein by large follicles exposed to GTH at Days 6-8 was not significantly different from the control group, the E_2 production of control and GTH-treated large follicles at Days 6-8 was significantly greater than at Days 30-32 (Fig. 15). The E_2 levels per mg of protein produced by untreated large follicles at Days 30-32 were similar to the levels produced by GTH-treated large follicles, with the exception of follicles exposed to 1 microgram GTH/ml which had greater E_2 production. The E_2 production per mg of protein by intermediate follicles was also relatively low throughout the 32-day experimental period. However, with the intermediate follicles, most of the GTH-treated groups were significantly elevated at both Days 6-8 and Days 30-32 compared to their respective untreated controls. Also, the E_2 level per mg of protein produced by intermediate follicles at Days 6-8 was greater than at Days 30-32 at the 1.0 and 0.05 microgram GTH/ml dose levels.

Mean GSI values of fish sampled throughout the 32-day experimental period were not significantly different from each other.

The results from Figs. 14 and 15 were, in part, transformed to mean values of E_2 and T produced per unit surface area (mm^2) of the follicles and are presented in Fig. 16. The E_2 production per unit surface area by large follicles that were untreated or exposed to GTH was greater on Days 6-8 than in the counterparts on Days 30-32; however, there were no significant differences between the GTH-treated and the untreated large follicles

Figure 15. Estradiol-17 beta (E_2) production per milligram of protein by large and intermediate ovarian follicles under varying doses of carp gonadotropin (0.01-1.0 microgram cGTH/ml) or in buffer alone (Control) after goldfish were acclimated for 6-8 and 30-32 days in 12L:12D/12°C in March, 1983. The gonadosomatic index (GSI) and the total number of fish killed (*n*) for the three consecutive sampling days are indicated. Ovarian follicles were sampled at different times during the day and each value, expressed as the mean±SEM of 10 to 25 replicates, represents data pooled from the three consecutive sampling days as outlined in Fig. 1.

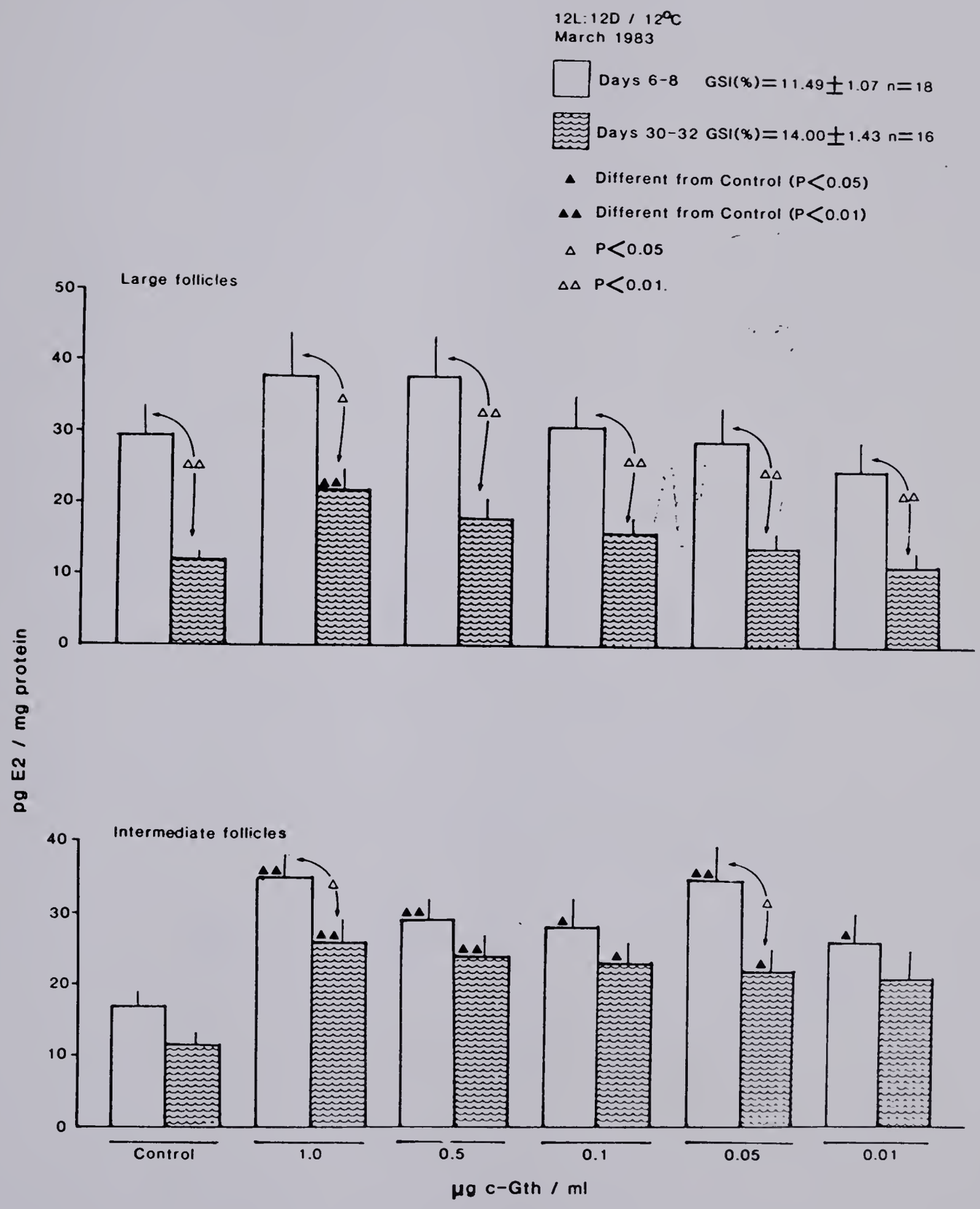
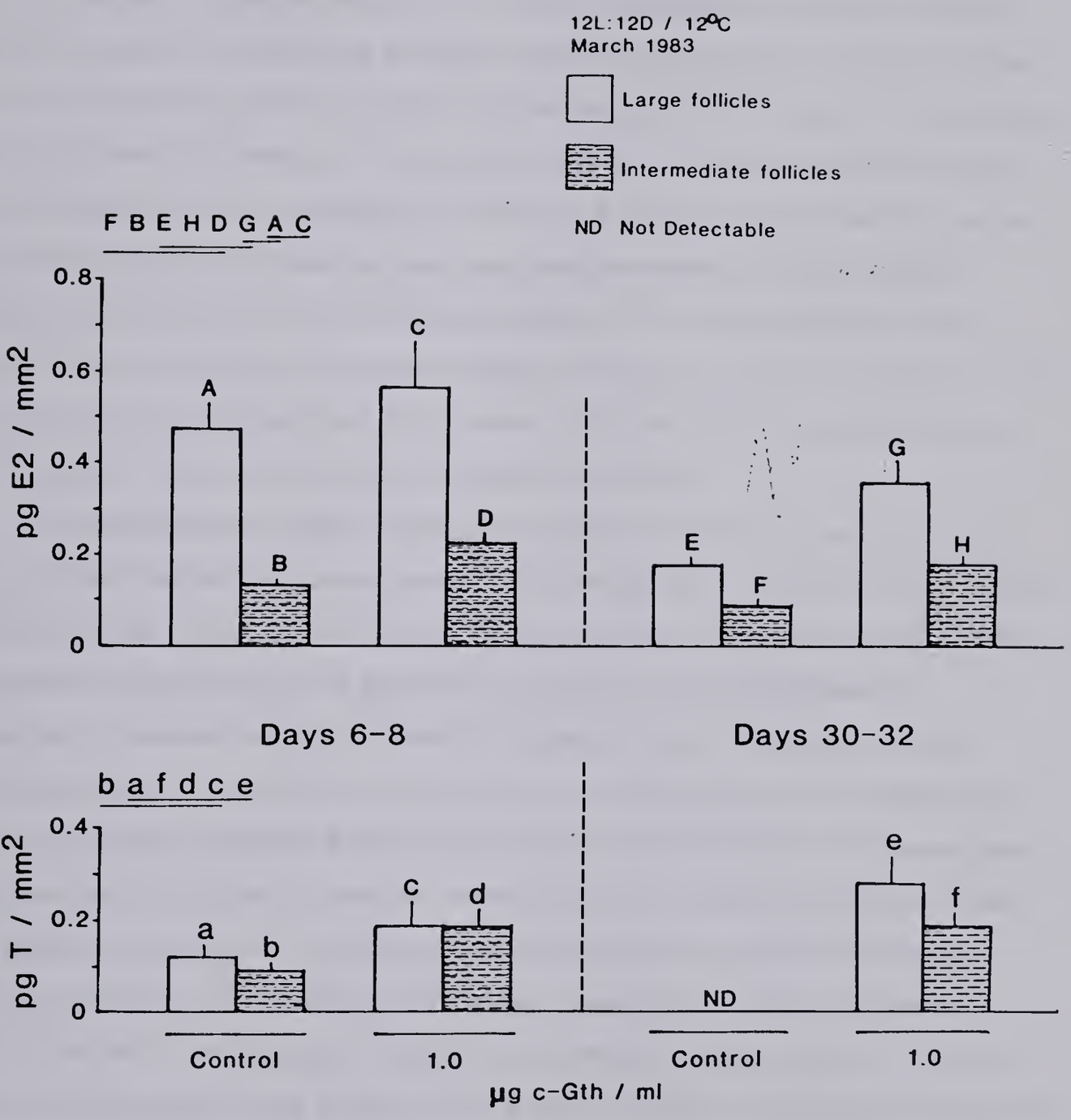

12L:12D / 12°C
March 1983
Days 6-8 GSI(%)=11.49±1.07 n=18
Days 30-32 GSI(%)=14.00±1.43 n=16
▲ Different from Control (P<0.05)
▲▲ Different from Control (P<0.01)
△ P<0.05
△△ P<0.01.
Large follicles
Intermediate follicles
pg E2 / mg protein
0
10
20
30
40
50
Control
1.0
0.5
0.1
0.05
0.01
µg c-Gth / ml

Figure 16. Estradiol-17 beta (E_2) and testosterone (T) production per unit surface area (mm^2) by carp gonadotropin-treated (1 microgram cGTH/ml) and untreated (Control) large and intermediate ovarian follicles from goldfish acclimated for 6-8 and 30-32 days in 12L:12D/12°C in March, 1983. Each value, expressed as the mean±SEM of 10 to 25 replicates, was transformed from data presented in Figs. 14 and 15. The results of a Duncan's multiple range test ($P<0.05$) are indicated; means which are not significantly different from each other are underlined.


12L:12D / 12°C
March 1983
Large follicles
Intermediate follicles
ND Not Detectable
F B E H D G A C
pg E2 / mm2
0.8
0.6
0.4
0.2
0
A
B
C
D
E
F
G
H
Days 6-8
Days 30-32
b a f d c e
pg T / mm2
0.4
0.2
0
a
b
c
d
ND
e
f
Control
1.0
Control
1.0
µg c-Gth / ml

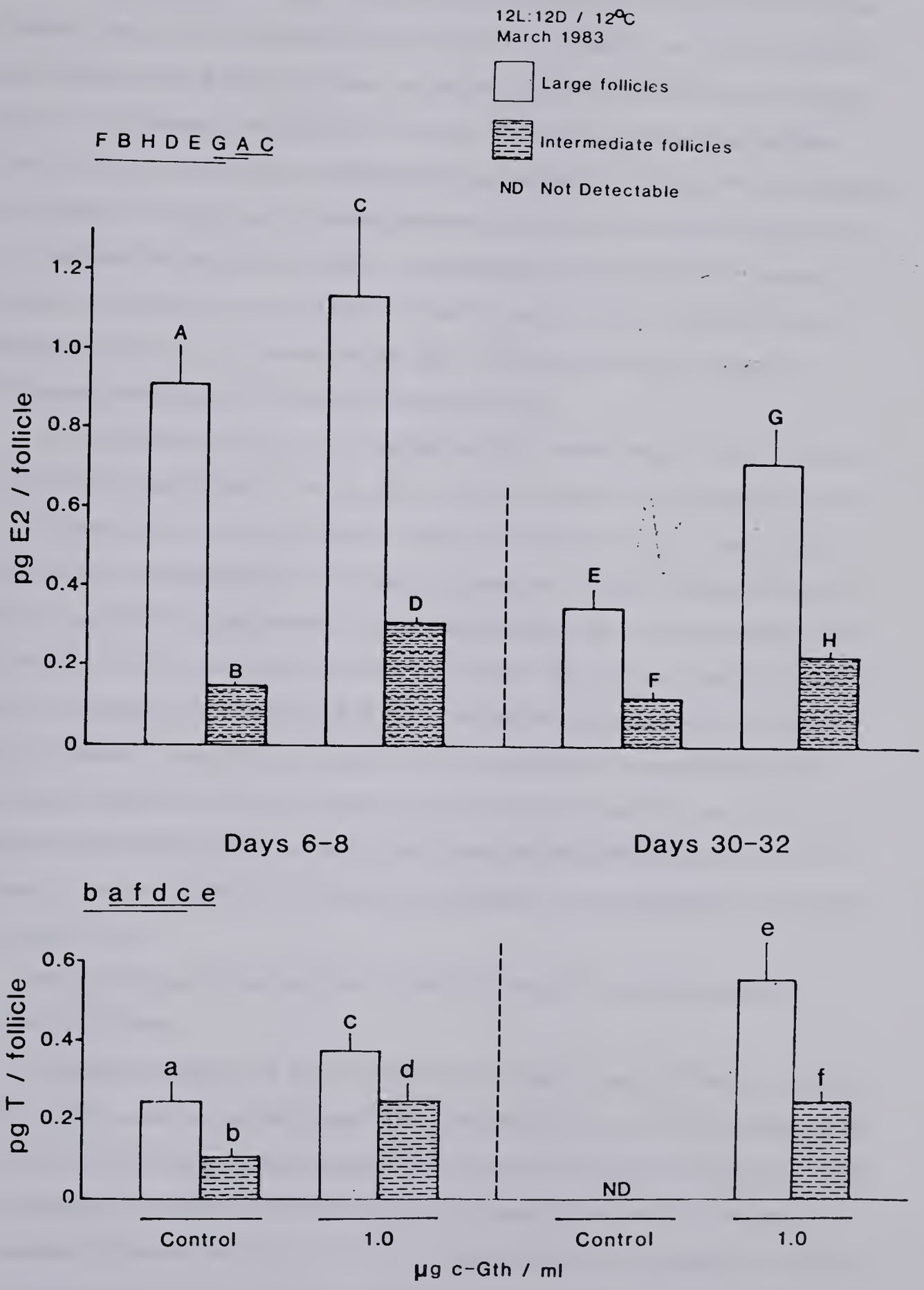
at Days 6-8. At Days 6-8 the E_2 production per unit surface area by GTH-treated and untreated large follicles were higher than by the GTH-treated and untreated intermediate follicles, respectively. Significant differences were observed in E_2 production per unit surface area between the GTH-treated large and intermediate follicles at Days 30-32. The E_2 production per unit surface area by GTH-treated and untreated intermediate follicles showed no significant differences throughout the experimental period. The E_2 production per unit surface area by GTH-treated and untreated large follicles, respectively, decreased after 30-32 days of acclimation. The production of T per unit surface area by both large and intermediate follicles exposed to GTH at Days 6-8 showed no significant differences from their respective untreated controls. Large and intermediate follicles incubated without GTH at Days 30-32 had no detectable levels of T per unit surface area. When treated with GTH, large and intermediate follicles had similar levels of T production per unit surface area at Days 6-8 and Days 30-32. Hence, GTH stimulated T production per unit surface area by large and intermediate follicles at Days 30-32.

The results shown in Figs. 14 and 15 were also transformed, in part, to E_2 and T production per follicle at the highest dose of GTH tested and are presented in Fig. 17. The E_2 production per follicle by large follicles treated with GTH was greater than GTH-treated intermediate follicles at Days 6-8 and 30-32; however, GTH did not stimulate E_2 production in large and intermediate follicles compared to their respective untreated controls at Days 6-8 and 30-32. The production of E_2 per follicle by GTH-treated large follicles was greater at Days 6-8 than at Days 30-32 in 12L:12D / 12°C. GTH-treated large follicles at Days 30-32 had E_2 levels per follicle similar to untreated large follicles at Days 6-8. Under GTH stimulation, T production per follicle between large and intermediate follicles did not show any significant differences throughout the entire experimental period. Likewise, T production per follicle by large and intermediate follicles did not show any significant variations after 6-8 and 30-32 days in 12L:12D / 12°C. At Days 30-32, GTH stimulated T production per follicle by large and intermediate follicles over their untreated controls which were not detectable.

b. June-July, 1983

Large follicles incubated without GTH did not produce detectable levels of T per mg of protein after 30-32 days of acclimation to 12L:12D / 12°C in June-July; however,

Figure 17. Estradiol-17 beta (E_2) and testosterone (T) production per follicle by carp gonadotropin-treated (1 microgram cGTH/ml) and untreated (Control) large and intermediate ovarian follicles from goldfish acclimated for 6-8 and 30-32 days in 12L:12D/12°C in March, 1983. Each value, expressed as the mean±SEM of 10 to 25 replicates, was transformed from data presented in Figs. 14 and 15. The results of a Duncan's multiple range test ($P<0.05$) are indicated; means which are not significantly different from each other are underlined.


12L:12D / 12°C
March 1983
Large follicles
Intermediate follicles
ND Not Detectable
F B H D E G A C
pg E2 / follicle
1.2
1.0
0.8
0.6
0.4
0.2
0
A
B
C
D
E
F
G
H
Days 6-8
Days 30-32
b a f d c e
pg T / follicle
0.6
0.4
0.2
0
a
b
c
d
ND
e
f
Control
1.0
Control
1.0
µg c-Gth / ml

incubation with GTH caused a significant stimulation of T output (Fig. 18). T produced per mg of protein by GTH-treated large follicles at Days 6-8 were not significant from their untreated controls. With or without GTH stimulation, T production per mg of protein by large follicles at Days 6-8 were greater than at Days 30-32 but only at the two lowest dosages of GTH tested. T produced per mg of protein by GTH-treated intermediate follicles at Days 6-8 were similar compared to their untreated controls with the exception of the highest GTH dose used. No detectable level of T per mg of protein was produced by intermediate follicles when incubated with buffer alone at Days 30-32; however, GTH stimulated T production at all dose levels tested. Except for their untreated controls, no significant differences were detected between T produced per mg of protein by GTH-treated intermediate follicles at Days 6-8 and 30-32.

The production of E_2 per mg of protein by GTH-treated large follicles at Days 6-8 was significantly higher than by the untreated controls, except at the highest GTH dose (Fig. 19). Levels of E_2 per mg of protein produced by untreated large follicles at Days 30-32 were not detectable; GTH stimulated E_2 production by large follicles at all dose levels at Days 30-32. Large follicles produced more E_2 per mg of protein on Days 6-8 than on Days 30-32, except at the 1 and 0.1 microgram GTH/ml dose levels, however, E_2 levels of untreated controls at Days 6-8 were greater than untreated controls at Days 30-32. Although E_2 production per mg of protein by most GTH-treated intermediate follicles was greater than by the respective control groups at Days 6-8 and 30-32, no significant differences were observed in the E_2 levels at Days 6-8 versus Days 30-32. However, E_2 levels produced by untreated intermediate follicles were higher at Days 6-8 than at Days 30-32.

Mean GSI values of fish sampled on Days 6-8 and on Days 30-32 were not significantly different.

The above data (Figs. 18 and 19) were transformed, in part, to T and E_2 production per unit surface area (mm^2) at the highest GTH dose tested (Fig. 20). The E_2 production per unit surface area by large and intermediate follicles treated with GTH were similar to each other throughout the experimental period. The E_2 production per unit surface area by intermediate follicles exposed to GTH at Days 6-8 and 30-32 were greater than by their respective untreated controls. GTH stimulated E_2 production per unit surface area by large

Figure 18. Testosterone (T) production per milligram of protein by large and intermediate ovarian follicles under varying doses of carp gonadotropin (0.01-1.0 microgram cGTH/ml) or in buffer alone (Control) after goldfish were acclimated for 6-8 and 30-32 days in 12L:12D/12°C in June-July, 1983. The gonadosomatic index (GSI) and the total number of fish killed (*n*) for the three consecutive sampling days are indicated. Ovarian follicles were sampled at different times during the day and each value, expressed as the mean±SEM of 10 to 30 replicates, represents data pooled from the three consecutive sampling days as outlined in Fig. 1.

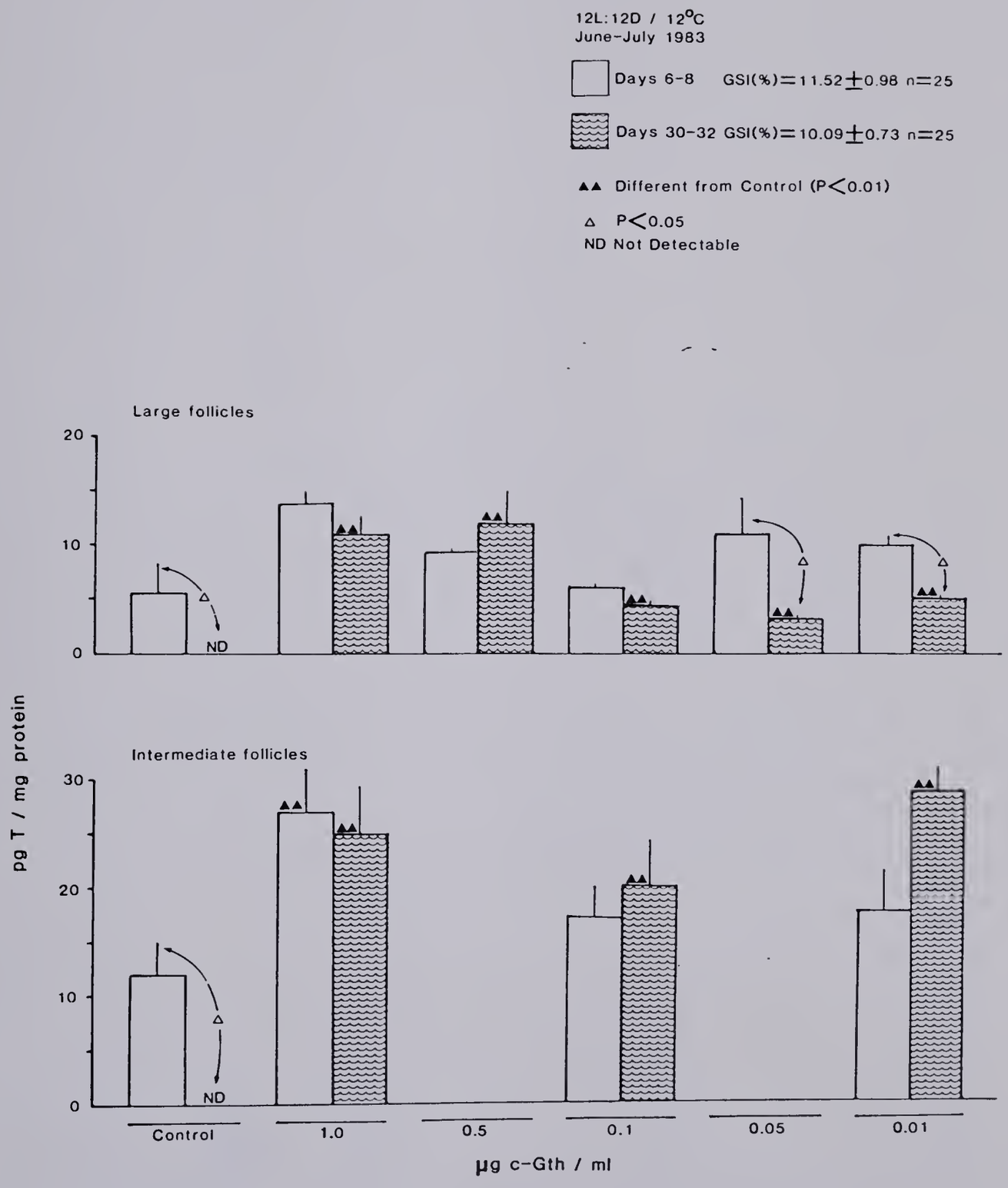

12L:12D / 12°C
June-July 1983
Days 6-8 GSI(%)=11.52±0.98 n=25
Days 30-32 GSI(%)=10.09±0.73 n=25
▲▲ Different from Control (P<0.01)
△ P<0.05
ND Not Detectable
Large follicles
Intermediate follicles
pg T / mg protein
ND
Control
1.0
0.5
0.1
0.05
0.01
µg c-Gth / ml

Figure 19. Estradiol-17 beta (E_2) production per milligram of protein by large and intermediate ovarian follicles under varying doses of carp gonadotropin (0.01-1.0 microgram cGTH/ml) or in buffer alone (Control) after goldfish were acclimated for 6-8 and 30-32 days in 12L:12D/12°C in June-July, 1983. The gonadosomatic index (GSI) and the total number of fish killed (*n*) for the three consecutive sampling days are indicated. Ovarian follicles were sampled at different times during the day and each value, expressed as the mean±SEM of 10 to 20 replicates, represents data pooled from the three consecutive sampling days as outlined in Fig. 1.

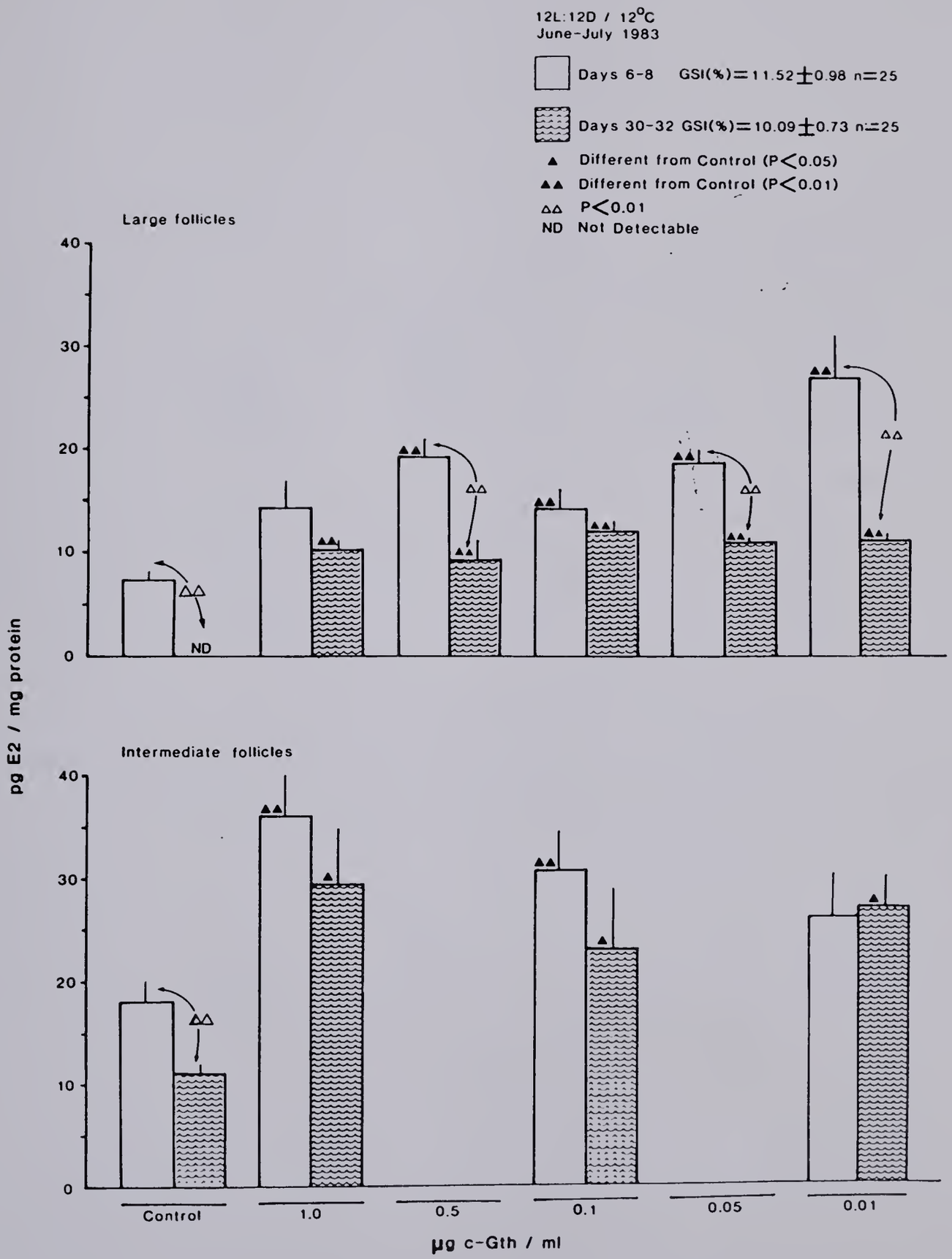
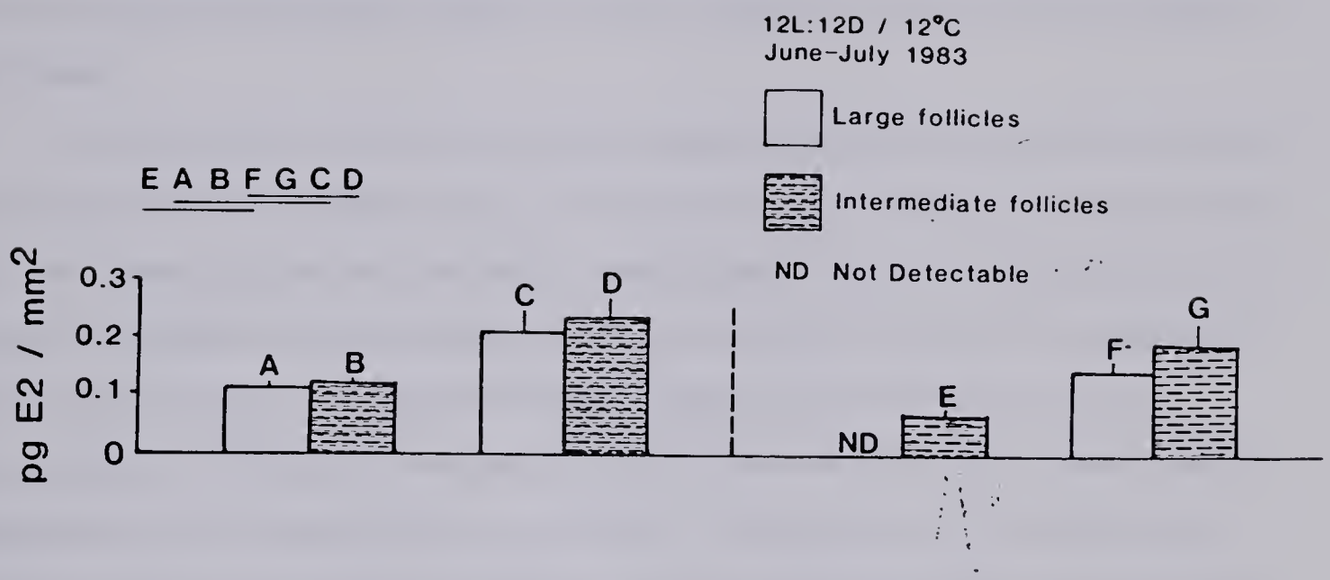
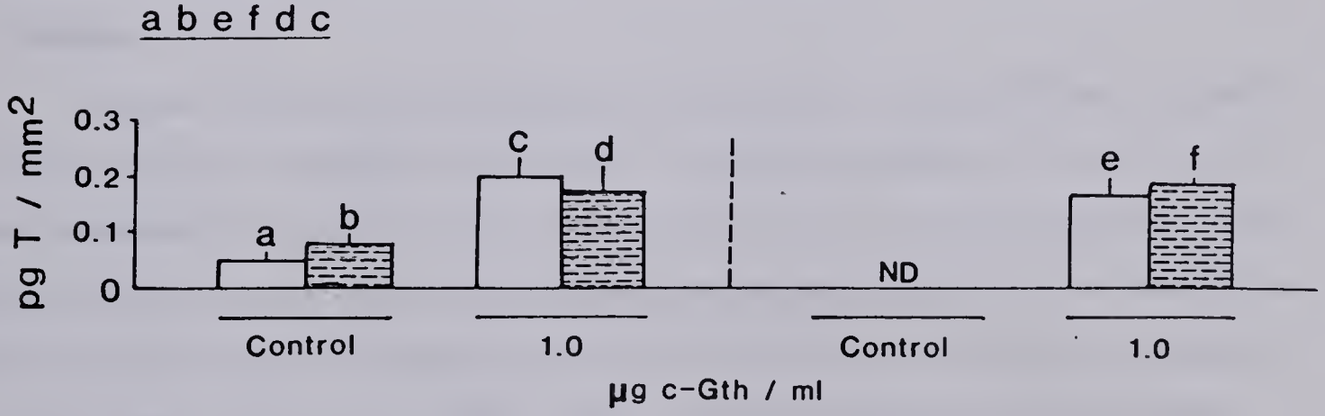

12L:12D / 12°C
June-July 1983
Days 6-8 GSI(%)=11.52±0.98 n=25
Days 30-32 GSI(%)=10.09±0.73 n=25
▲ Different from Control (P<0.05)
▲▲ Different from Control (P<0.01)
△△ P<0.01
ND Not Detectable
Large follicles
Intermediate follicles
pg E2 / mg protein
ND
0
10
20
30
40
Control
1.0
0.5
0.1
0.05
0.01
µg c-Gth / ml

Figure 20. Estradiol-17 beta (E_2) and testosterone (T) production per unit surface area (mm^2) by carp gonadotropin-treated (1 microgram cGTH/ml) and untreated (Control) large and intermediate ovarian follicles from goldfish acclimated for 6-8 and 30-32 days in 12L:12D/12°C in June-July, 1983. Each value, expressed as the mean±SEM of 10 to 30 replicates, was transformed from data presented in Figs. 18 and 19. The results of a Duncan's multiple range test ($P<0.05$) are indicated; means which are not significantly different from each other are underlined.


12L:12D / 12°C
June-July 1983
Large follicles
Intermediate follicles
ND Not Detectable
E A B F G C D
pg E2 / mm2
0.3
0.2
0.1
0
A
B
C
D
ND
E
F
G
Days 6-8
Days 30-32


a b e f d c
pg T / mm2
0.3
0.2
0.1
0
a
b
c
d
ND
e
f
Control
1.0
Control
1.0
µg c-Gth / ml

follicles compared to their untreated controls at Days 6-8. The production of T per unit surface area by both GTH-treated and untreated large and intermediate follicles were low throughout the 32-day experimental period. No significant stimulation by GTH of T production per unit surface area by large and intermediate follicles were noted over their untreated controls at Days 6-8. However, GTH stimulated T production per unit surface area by large and intermediate follicles over their untreated controls after 30-32 days of acclimation.

The data in Figs. 18 and 19 were also transformed, in part, to E_2 and T production per follicle at the highest dose level of GTH tested (Fig. 21). The E_2 production per follicle by GTH-treated large follicles were significantly higher than by their respective untreated controls throughout the experimental period. Only at Days 30-32 was E_2 produced per follicle by GTH-treated intermediate follicles greater than its untreated control. E_2 production per follicle by GTH-exposed large and intermediate follicles, respectively, at Days 6-8 and 30-32 were similar to each other. On the other hand, T production per follicle by large and intermediate follicles exposed to GTH at Days 6-8 were significantly different from each other. Compared to their untreated controls, GTH stimulated T production per follicle by large follicles throughout the experimental period. GTH stimulated T production per follicle by intermediate follicles at Days 30-32 only.

C. Effect of Incubation Temperature on Steroid Production by Goldfish Ovarian Follicles *In Vitro*

Ovarian follicles from fish acclimated for 8 days at 16L:8D / 20°C and sampled at 1100h in January, 1983 (GSI=5.72±0.49%, n=13) were incubated for 4 hours either at their acclimation temperature of 20°C or at 12°C (Fig. 22). Under GTH treatment, T and E_2 production per mg of protein for all follicle sizes was significantly lower at 12°C than at 20°C. Ovarian follicles from fish acclimated at 12L:12D / 12°C for 8 days and sampled at 1100h in March, 1983 (GSI=13.39± 2.36%, n=3) were incubated at their acclimation temperature of 12°C or at 20°C for 4 hours (Fig. 23). Similarly, results illustrated in Fig. 23 show that there was significantly less T and E_2 production per mg of protein at 12°C under GTH treatment for all follicle sizes.

Figure 21. Estradiol-17 beta (E_2) and testosterone (T) production per follicle by carp gonadotropin-treated (1 microgram cGTH/ml) and untreated (Control) large and intermediate ovarian follicles from goldfish acclimated for 6-8 and 30-32 days in 12L:12D/12°C in June-July, 1983. Each value, expressed as the mean±SEM of 10 to 30 replicates, was transformed from data presented in Figs. 18 and 19. The results of a Duncan's multiple range test ($P<0.05$) are indicated; means which are not significantly different from each other are underlined.

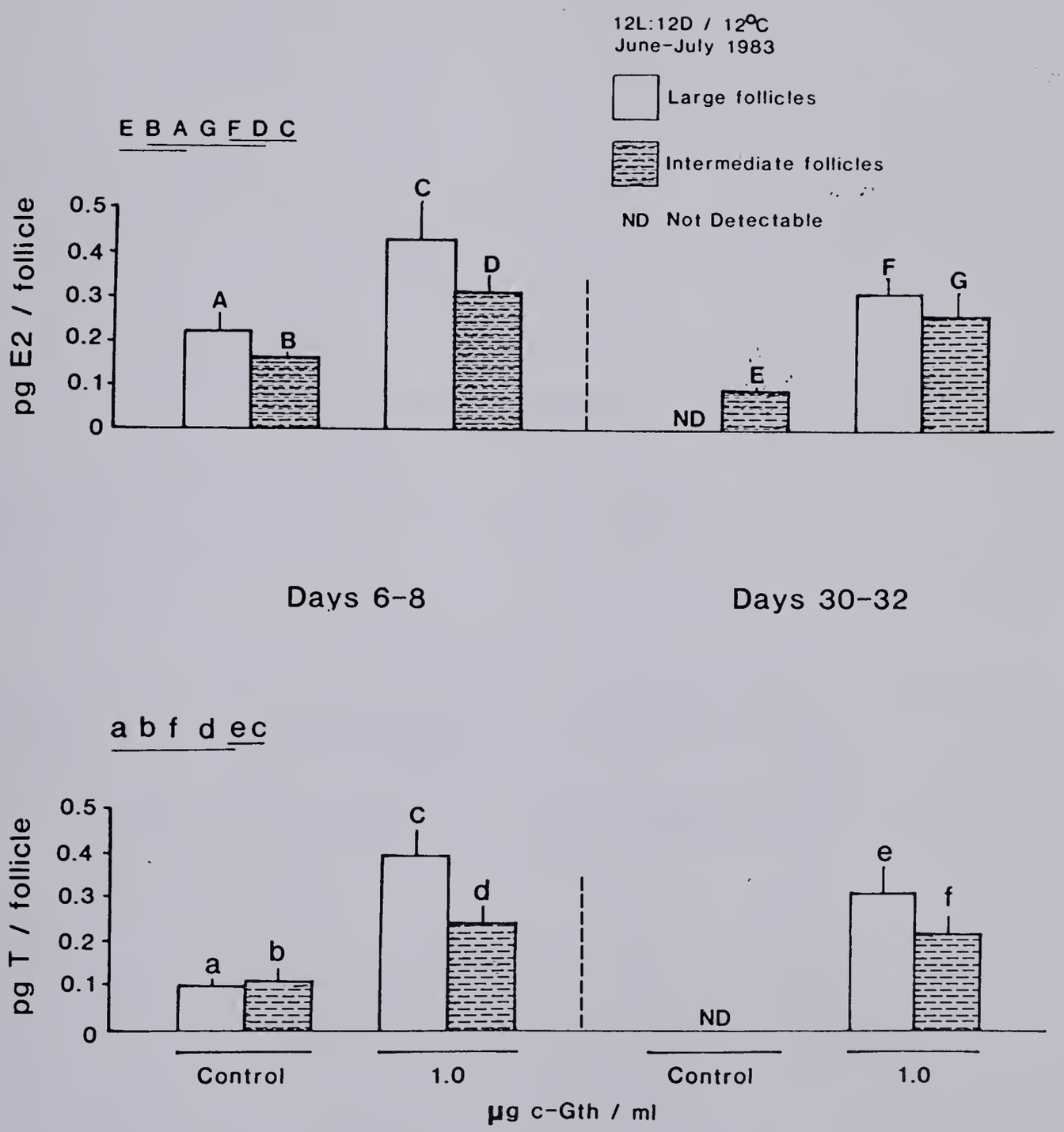

12L:12D / 12°C
June-July 1983
Large follicles
Intermediate follicles
ND Not Detectable
E B A G F D C
pg E2 / follicle
0.5
0.4
0.3
0.2
0.1
0
A
B
C
D
E
ND
F
G
Days 6-8
Days 30-32
a b f d e c
pg T / follicle
0.5
0.4
0.3
0.2
0.1
0
a
b
c
d
ND
e
f
Control
1.0
Control
1.0
µg c-Gth / ml

Figure 22. The effect of incubation temperature on the estradiol-17 beta (E_2) and testosterone (T) production per milligram of protein by carp gonadotropin-treated (0.01-1.0 microgram cGTH/ml) and untreated (Control) large, intermediate and small ovarian follicles from goldfish sampled at 1100h on Day 8 of acclimation at 16L:8D/20°C in January, 1983 [Gonadosomatic index (GSI) = 5.72±0.49%, *n* = 13 fish]. Ovarian follicles were incubated for 4 hours either at 20°C or at 12°C. Each value represents the mean±SEM of three to five replicates.

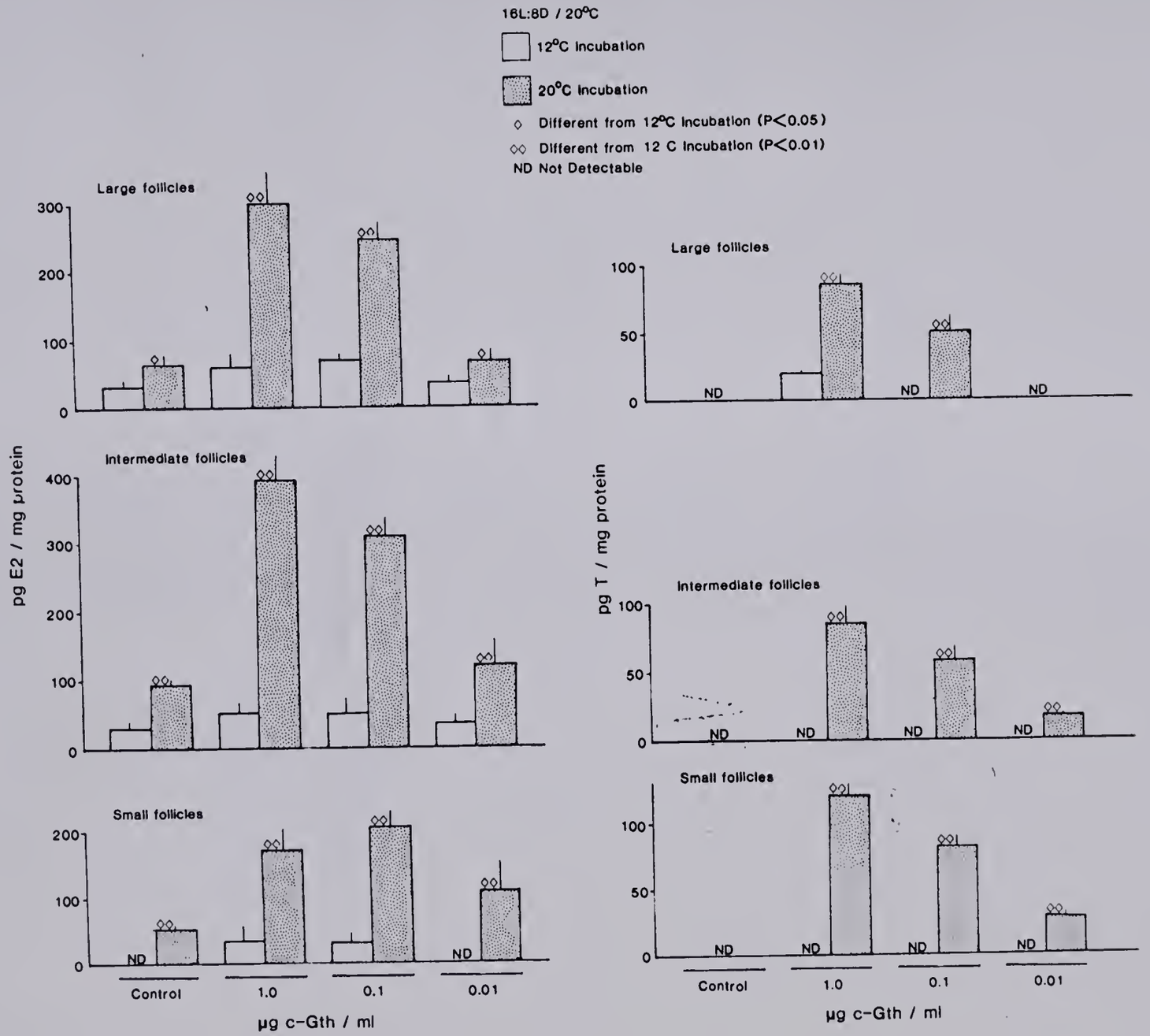
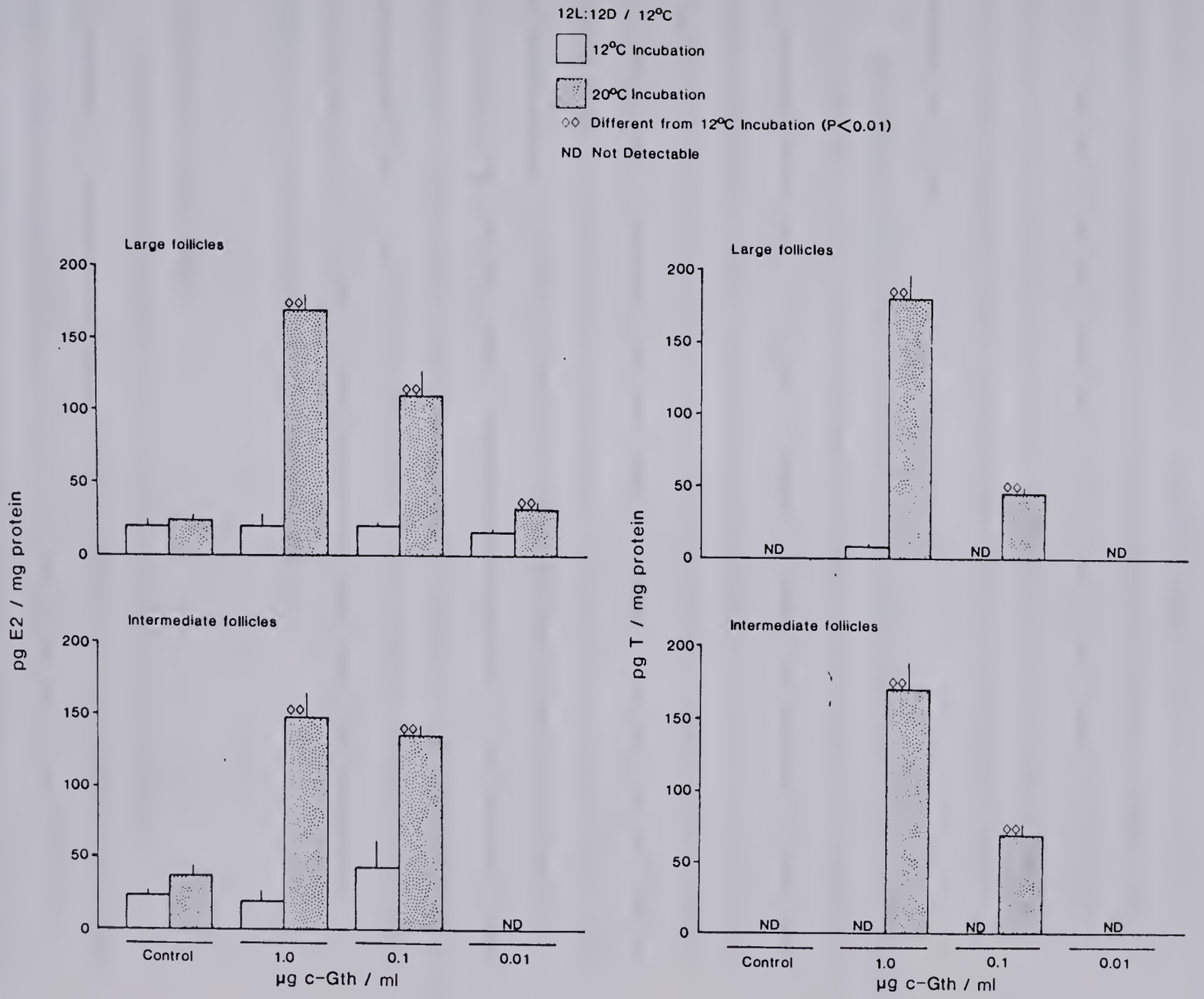

16L:8D / 20°C
12°C Incubation
20°C Incubation
◇ Different from 12°C Incubation (P<0.05)
◇◇ Different from 12 C Incubation (P<0.01)
ND Not Detectable
Large follicles
Intermediate follicles
Small follicles
pg E2 / mg protein
pg T / mg protein
ND
Control
1.0
0.1
0.01
µg c-Gth / ml

Figure 23. The effect of incubation temperature on the estradiol-17 beta (E_2) and testosterone (T) production per milligram of protein by carp gonadotropin-treated (0.01-1.0 microgram cGTH/ml) and untreated (Control) large and intermediate ovarian follicles from goldfish sampled at 1100h on Day 8 of acclimation at 12L:12D/12°C in March, 1983 [Gonadosomatic index (GSI) = 13.39±2.36%, *n* = 3 fish] Ovarian follicles were incubated for 4 hours either at 12°C or at 20°C. Each value represents the mean±SEM of three to five replicates.


12L:12D / 12°C
12°C Incubation
20°C Incubation
◊◊ Different from 12°C Incubation (P<0.01)
ND Not Detectable
Large follicles
Intermediate follicles
pg E2 / mg protein
pg T / mg protein
200
150
100
50
0
ND
Control
1.0
0.1
0.01
µg c-Gth / ml

IV. DISCUSSION

The existence of variations in the steroidogenic capacity, in terms of estradiol- 17 beta (E_2) and testosterone (T) production, of goldfish ovarian follicles *in vitro* was investigated in the present study. At two different times in the season ("winter" and "spring"), ovarian follicles were sampled diurnally from fish acclimated for different lengths of time to 12L:12D / 12°C or 16L:8D / 20°C, after an initial 8 day period of 12L:12D / 12°C, and subsequently incubated with varying concentrations of carp gonadotropin (cGTH).

The results of the present investigation were expressed as pg of steroid produced per mg of protein, per unit surface area and per follicle. These methods of presenting the data provide several ways of comparing the steroid production between different sizes of follicles, although each method may show entirely different profiles of the same steroid production level. Steroid production expressed on a per mg of protein or on a weight basis is important in determining the steroidogenic capacity of a given mass of follicles, since the amount of yolk proteins incorporated by the oocytes varies significantly during the breeding season. The transformation of part of the data to steroid levels per unit surface area of the follicles is also of importance because the total surface area of each follicle changes significantly through the course of oocyte growth and development; such an assessment has not been done previously for teleost oocytes. The data expressed as steroid production per follicle is a direct method of determining the steroidogenic capacity of each follicle size during oocyte development.

A. Short-Term Experiments

After an initial acclimation to a 12L:12D / 12°C regime for 8 days, the environmental conditions were changed to 16L:8D / 20° C; 5-7 days later the fish were killed at 1100h and 1900h and the ovarian follicles incubated with GTH at 20°C for 4 hours. This experiment was performed on "winter" (January, 1983) and on "spring" (April, 1983) fish. Regardless of follicle size, follicles from "winter" fish showed a daily variation in responsiveness to at least some dosages of GTH, with follicles sampled at 1100h

producing more T and E_2 per mg of protein than follicles sampled at 1900h. The difference was most prominent with the intermediate follicles where E_2 levels produced under GTH stimulation at 1100h were close to three times the levels produced at 1900h. On the other hand, the steroid production by GTH-treated ovarian follicles from "spring" fish either lacked a significant variation in steroid production between the two sampling times, or exhibited an inverted mode of response, wherein follicles from fish killed at 1900h had higher production of E_2 and T than follicles from fish sampled at 1100h; notably, this significant difference was limited to large follicles only. T levels produced per mg of protein by all follicle sizes remained relatively constant in "winter" and in "spring" follicles.

Although the results do not demonstrate a diurnal rhythm of steroid secretion by ovarian follicles *in vitro* since the sampling times were limited to only two times of the day, the data clearly indicate that the steroidogenic responsiveness to GTH varies throughout the day, and support the hypothesis suggested by Peter *et al.* (1982) that a daily cycle of ovarian GTH receptors exists in the teleost ovary. This hypothesis is indirectly supported by the findings that there is an accumulation of cAMP in the mullet ovary in response to GTH and prostaglandin treatments *in vitro* at several hours after lights-on but not at other times (Kuo and Watanabe, 1978), a significant daily fluctuation of serum GTH in the goldfish (Hontela and Peter, 1978; Hontela, 1982), a temporal responsiveness of the gonads of the golden shiner to injections of salmon GTH (sGTH) and luteinizing hormone (LH) (de Vlaming and Vodicnik, 1977), a temporal responsiveness of the goldfish ovary to sGTH and cGTH (Peter *et al.*, 1982), and the daily surges of plasma T and E_2 in the Indian catfish (Lamba *et al.*, 1983).

As demonstrated by Hontela and Peter (1978) and Hontela (1982), recrudescing female goldfish in January have a GTH variation of greater magnitude than mature fish in March under the same environmental conditions (16L:8D / 20°C) and feeding regimes used in the present investigation. In the present study, all sizes of follicles from "winter" fish produced significantly greater amounts of T and E_2 per mg of protein at 1100h than at 1900h to most dosages of GTH. It is possible that follicles from "winter" or recrudescing female goldfish would be producing more steroids in response to the high mid-day surge of circulating GTH known to occur in fish at this particular stage of sexual maturity when

held under the given environmental conditions. Hence, it is likely that there is a close synchrony of ovarian GTH receptors to the daily cycle of pituitary GTH release. A distinct advantage of this synchrony would be a greater stimulation of ovarian development since more receptors would be available at the time when circulating GTH levels are highest. Indeed, Hontela and Peter (1978) and Hontela (1982) suggested that fluctuating GTH levels may be important for stimulation of goldfish ovarian development and that the optimization of ovarian growth occurs when the daily peak in GTH levels coincides with the daily peak of responsiveness of the ovaries. E_2 and T together with GTH may possibly act on the ovary to influence its responsiveness. E_2 has been shown to increase the level of follicular responsiveness to GTH by enhancing the appearance of granulosa cell LH receptors in the mammalian ovary (Richards, 1979). In goldfish acclimated for 5-6 days in 16L:8D / 20°C, B. Breton, R.E. Peter and C.S. Nahorniak (unpublished results) demonstrated *in vitro* that receptors can be induced by GTH in large, preovulatory follicles and not in smaller follicles. It remains to be shown whether the mechanism of receptor induction or activation in the teleost ovary resembles the mammalian model which involves gonadal steroids acting synergistically with GTH.

Hontela (1982) showed that female goldfish kept for 5-7 days at 16L:8D / 20°C in March had low and constant serum levels of GTH throughout the day; only after the fish were maintained for 11-13 days under these conditions was a significant mid-day peak (1100h) in serum GTH levels observed. Also, the amplitude of the daily variation in serum GTH levels found in "winter" fish by Hontela and Peter (1978) and Hontela (1982) was much greater than the amplitude found in "spring" fish. The present data which showed that intermediate-sized follicles from "spring" fish produced uniform T and E_2 levels per mg of protein at 1100h and 1900h, and lower amounts of steroid compared to the "winter" follicles, may be related to the above observations on serum GTH levels. It may be that relatively constant or low magnitude variations in GTH levels during the day lead to a low level of responsiveness by the ovaries. Whether the lower responsiveness by ovarian follicles from "spring" fish compared to "winter" fish is due to a lower receptor population or a lower binding of GTH to these follicular receptors remains to be investigated.

Kagawa *et al.*(1981, 1983a) have demonstrated a good correlation of T and E_2 production between isolated ovarian follicles *in vitro* and plasma steroid levels in the

amago salmon and the white-spotted char during their annual reproductive cycles. Likewise, Zohar *et al.* (1982) demonstrated that by subjecting fragments of rainbow trout ovaries to GTH pulses in an open perifusion system fragments taken from fish undergoing the peak of exogenous vitellogenesis had the longest (16 hours) extended amplification of E_2 output compared to ovarian fragments from fish in early recrudescence or in a mature stage. Under these conditions, ovarian fragments from an early recrudescing rainbow trout showed an immediate and continuous E_2 output that gradually decreased over 8 hours; on the other hand, fragments from mature fish showed an increased E_2 output which rapidly declined after GTH stimulation. Taken together, these studies suggest that ovaries of salmonid species show an elevated steroidogenic responsiveness during the period of rapid oocyte growth. The present data from goldfish also fit with this pattern in that the period of most active vitellogenesis is in the "winter" fish , and the follicles from "winter" fish had the greatest E_2 production per mg of protein in response to GTH stimulation. It is somewhat incongruous that intermediate follicles apparently in the middle of vitellogenesis from "spring" fish were less responsive than "winter" follicles of the same size. However, in the Long-Term Experiment, T and E_2 production per mg of protein, per unit surface area and per follicle by intermediate follicles under the highest GTH dose tested were relatively similar in "winter" and in "spring" after 5-7 days of exposure to 16L:8D / 20°C. The time of sampling of the fish in early January in the Short-Term Experiment, in contrast to sampling the follicles in late January and early February in the Long-Term Experiment, could have contributed to the higher E_2 levels produced by intermediate "winter" follicles in the Short-Term Experiment.

Although the frequencies of the different follicle sizes remained the same, the higher T and E_2 production by large "spring" follicles sampled at 1900h compared to 1100h may be attributed to the more advanced stage of maturity of these follicles in terms of the ability of GTH to induce its own specific receptors *in vitro* (B. Breton, R.E. Peter and C.S. Nahorniak, unpublished data). These large follicles are possibly at a preovulatory stage awaiting the ovulatory surge of GTH. This may entail a shift in the time of the steroidogenic responsiveness of large, preovulatory follicles to just prior to the onset of scotophase (1900h) enabling them to better respond to the the ovulatory GTH surge when it occurs under the conditions described by Stacey *et al.* (1979a,b). In rats, a

progressive increase in ovarian responsiveness to LH coincides with an increased LH receptor content in the granulosa cells of preovulatory follicles, resulting in an increased production of E_2 which triggers the ovulatory surge of LH (Uilenbroek and van der Linden, 1983). Also, in the rat testis, elevated serum GTH levels caused an increase in the number of testicular LH receptors, although receptor occupancy by LH/HCG was at a minimal level (Powell *et al.*, 1981).

The relatively lower levels of T production per mg of protein compared to E_2 produced by "winter" and "spring" follicles is also in good agreement with the substrate-product relationship existing between T and E_2. Similar to the two-cell type mammalian model, Kagawa *et al.* (1982) have demonstrated in the amago salmon that testosterone is specifically synthesized in the theca cells and is transported intercellularly to the granulosa cell layer where it is aromatized to E_2. Therefore, an increased production of E_2 by the follicles leads to a reduction of the substrate (T).

It is interesting to speculate whether the ovarian follicles would also exhibit a temporal responsiveness to the GTH molecule with a low carbohydrate content (Con A-I) or whether the follicles are differentially responsive to each type of GTH molecule. The GTH used in the present study was the Con A-II fraction which has a relatively higher carbohydrate content and is capable of inducing final oocyte maturation, ovulation, spermiation, cAMP accumulation and steroidogenesis, whereas Con A-I GTH can stimulate vitellogenin uptake and steroidogenesis (Idler and Ng, 1979; Ng and Idler, 1979). The present work has shown that there is a daily change in responsiveness of all follicle sizes to the Con A-II GTH, but whether Con A-I GTH is also capable of eliciting similar results is not known.

B. Long-Term Experiments

In this series of experiments , the effects of different lengths of acclimation of female goldfish in "winter" and in "spring" to a 16L:8D/20°C and a 12L:12D/12°C condition on the steroidogenic capacity of ovarian follicles were tested.

The effects of exposure to warm temperature and long photoperiod are dependent on the length of exposure, the sexual stage of the fish and the season when these conditions are applied. Previous work on the golden shiner by de Vlaming (1975),

the Indian catfish by Vasal and Sundararaj (1976) and the goldfish by Gillet *et al.* (1978) showed a marked development of recrudescing and mature ovaries after a long-term exposure to warm water. Similarly, Hontela and Peter (1978) demonstrated a stimulatory effect of warm water and long photoperiod on recrudescing female goldfish within 7-9 days of exposure and noted a significant daily fluctuation in serum GTH levels concomitant with this effect. In the present investigation, GTH-treated large follicles from "winter" and "spring" fish generally had higher steroid production levels at Days 5-7 than at Days 11-13 and 30-32 of acclimation to 16L:8D / 20°C. The E_2 production per mg of protein , per unit surface area and per follicle by GTH-treated large "winter" follicles at Days 11-13 and 30-32 were generally similar; however, only after 30-32 days of acclimation was T production per unit surface area and per follicle similar to the high levels observed at Days 5-7, indicating that both a short-term and a long-term exposure of recrudescing fish to 16L:8D / 20°C in winter promotes steroidogenesis in large follicles under GTH stimulation. The higher T and E_2 outputs per unit surface area and per follicle by GTH-treated large "winter" and "spring" follicles usually found relative to follicles of smaller diameter could contribute to the maintenance and promotion of oocyte growth especially evident in intermediate "winter" and "spring" follicles (see discussion below).

Under GTH stimulation, large "spring" follicles generally showed a progressive decline in steroid production expressed per mg of protein, per unit surface area or per follicle throughout the experimental period at 16L:8D / 20°C. This observation is most apparent with T production per mg of protein by these follicles. However, E_2 production per mg of protein at Days 5-7 was always higher than at Days 30-32 but not at Days 11-13; only at 0.5 and 0.1 microgram GTH / ml doses was E_2 production per mg of protein at Days 11-13 lower than levels at Days 5-7. Notably, there was no significant stimulation by GTH of E_2 production per mg of protein by large "spring" follicles at Days 30-32. Likewise, E_2 production per mg of protein by large "spring" follicles under the highest GTH dose level was five times less than by similar sized follicles in "winter". Since these follicles were sampled from sexually mature fish in "spring", the general progressive decrease in T and E_2 levels produced per mg of protein, per unit surface area and per follicle by large follicles suggests that large follicles from mature fish held on long photoperiod and warm water conditions can maintain E_2 and T production capability for a

number of days before a decline in responsiveness occurs. This may explain why a long-term application of warmth to female goldfish in November and March resulted in high and uniform serum GTH levels throughout the day and was accompanied by a high incidence of ovarian regression (Gillet *et al.*, 1978; Hontela, 1982). The decline in E_2 production per mg of protein, per unit surface area and per follicle by large "spring" follicles treated with GTH at the end of the 32-day experimental period may also be an indication that ovarian GTH receptors are inactivated after a long-term exposure to the experimental conditions or are lost as the fish approaches a prespawning condition. The shift in E_2 production may also reflect an effect of temperature on the activity of ovarian follicular enzymes. Kime (1982) found an increase in the biosynthesis of steroid glucuronides in fish testes *in vitro* at warm temperatures, which may explain the failure of rainbow trout to spermiate *in vivo* at elevated temperatures. However, no seasonal correlation has been detected between glucuronide synthesis and ambient water temperature *in vivo*; the series of investigations by Kime (1982) have shown that *in vitro* activation of glucuronyl transferase in the testes and the liver of brown trout, rainbow trout and the goldfish occurs at temperatures above those which are the environmentally preferred temperatures for reproduction by these species.

It is also possible that the overall low level of responsiveness in terms of E_2 production per mg of protein, per unit surface area and per follicle by large "spring" follicles in contrast to large "winter" follicles can be attributed to preparation for the ovulatory response to GTH in "spring". Young *et al.* (1983a), Kagawa *et al.* (1983a), Nagahama and Kagawa (1982) and Kagawa *et al.*(1984) have interpreted a decrease in *in vitro* E_2 production by mature follicles of amago salmon and goldfish as evidence of an inhibition of aromatase enzyme activity prior to ovulation. The increasing titres of plasma GTH in preovulatory rainbow trout has been suggested by Scott *et al.* (1983) to suppress C21→C19 desmolase activity and enhance the activity of 20 beta-HSD oxidoreductase leading to the production of the maturation-inducing steroid (17 alpha,20 beta-P). The ability of a salmon maturational GTH to inhibit aromatase enzyme activity *in vitro* was also demonstrated by Sire and Depeche (1981) but on pre- and early vitellogenic ovaries of rainbow trout. Therefore, an extended exposure to warmth and long photoperiod of mature goldfish in "spring" may lead to preparation for the ovulatory response, by shifting

follicular production from C19 steroids to C21 steroids. The high and constant serum levels of GTH during the day in female goldfish in "spring" observed by Hontela (1982) after 30-32 days of acclimation to 16L:8D / 20°C may possibly serve a similar purpose as suggested by Scott *et al.* (1983) in the rainbow trout in suppressing C21→ C19 desmolase activity in large preovulatory follicles from "spring" fish. Furthermore, in the goldfish, Stacey *et al.* (1983) measured 17 alpha, 20 beta-P at 5 hours prior to ovulation in brain-lesioned goldfish, suggesting that a transient GTH stimulation of the progestogen pathway occurs, further supporting this interpretation. However, this line of reasoning does not account for the higher incidence of oocyte regression under long-term exposure to 16L:8D / 20°C (Hontela, 1982), which leads to the suggestion given above that the decline in steroid production observed resulted from a decreased responsiveness to GTH due to some change in receptors. On the basis of the present data, it is not possible to favor one hypothesis over another; it is possible that both situations occur at the same time.

Although E_2 output per follicle and per unit surface area by intermediate "winter" follicles remained constant throughout the 32-day experimental period at 16L:8D / 20°C, E_2 production per mg of protein under GTH stimulation at Days 11-13 were generally lower in comparison to levels at Days 5-7 and 30-32. No apparent explanation could account for these differences; however, the continuous output of E_2 expressed per unit surface area and per follicle by intermediate "winter" follicles treated with GTH indicates a constant level of responsiveness to GTH stimulation of goldfish ovaries in a recrudescing stage. Similarly, ovarian fragments from vitellogenic rainbow trout (early ovarian recrudescence and exogenous vitellogenic stage) showed an amplification and prolonged enhancement of E_2 secretion *in vitro* following the application of a GTH pulse (Zohar *et al.*, 1982), supporting their hypothesis that daily fluctuations of GTH levels act to stimulate E_2 secretion and ovarian development in this species. Hence, the continuous production of E_2 per unit surface area and per follicle by intermediate follicles from "winter" goldfish under the influence of GTH likely contributes to the maintenance of vitellogenesis in the recrudescing goldfish ovary. Khoo (1979) had, in fact, indicated that estrogens induced the formation of yolk vesicles while pregnenolone induced the formation of yolk granules in hypohysectomized goldfish.

While E_2 production per mg of protein by GTH-exposed intermediate "spring" follicles were similar and high at Days 5-7 and 11-13 compared to levels at Days 30-32, E_2 output per unit surface area and per follicle were higher at Days 11-13 than at Days 5-7 and 30-32; the lowest levels of E_2 production per unit surface area and per follicle by GTH-treated intermediate follicles in "spring" were also observed at Days 30-32. This suggests that a long-term exposure to 16L:8D / 20°C in "spring" decreases the responsiveness of these follicles to GTH stimulation as determined by E_2 production. The decline in E_2 output per mg of protein, per unit surface area and per follicle by both large and intermediate follicles in "spring" in response to GTH at Days 30-32 could also partly explain the occurrence of higher proportions of atretic follicles observed by Hontela (1982). However, the ability of GTH to stimulate T and E_2 production by large "spring" follicles could probably sustain the development of yolky oocytes during the spawning months by providing androgens for aromatization to estrogens by smaller-sized follicles.

The results presented in Figs. 8 and 9 show E_2 and T production by all follicle size groups expressed per unit surface area or per follicle from "winter" fish kept at 16L:8D / 20°C. On a per unit surface area and a per follicle basis, large "winter" follicles produced more T and E_2 compared to the other follicle sizes throughout the 32-day experimental period. As previously stated, the data expressed on a per unit surface area or on a per follicle basis allow a more direct method of comparing steroid production capacity between different sizes of follicles. It may at first seem from the present data that large "winter" follicles account for a large majority of the circulating T and E_2 pool as this particuar size group was more responsive to GTH stimulation compared to the smaller follicles. However, in an analysis of the cellular composition of goldfish ovaries at an early stage of ovarian recrudescence (GSI=3.4%-3.1%) in November after subjecting fish to similar environmental conditions used in the present investigation, Hontela (1982) found that the ovaries consisted mostly of what are termed here "intermediate follicles" or oocytes at the yolk vesicle and at the primary and secondary yolk globule stages. Tertiary yolk globule oocytes ("large-sized follicles" in the present study) were rarely observed in the goldfish ovaries at this time of the year. Although the fish used in the present study were at a later stage of ovarian recrudescence (GSI= 7.34%-4.34%) in January-February, and may have had a slightly different ovarian cellular composition than the goldfish ovaries

in the study by Hontela (1982), it is still apparent that the relative contribution of intermediate and small follicles to circulating T and E_2 in "winter" goldfish must be greater than by the more responsive large follicles due to the greater number and the larger combined surface area of the former. A sharp increase in plasma E_2 levels of female goldfish held under natural environmental conditions in January-February was observed by Kagawa *et al.* (1983b), supporting the present suggestion that an accelerated recruitment of intermediate and small follicles is reflected by a gradual increase in GSI in January-February, and the accompanying increase in surface area of follicles results in greater steroid production.

As shown in Figs. 12 and 13, large "spring" follicles produced significantly more E_2 per follicle than intermediate follicles at Days 5-7 only and, in terms of T production per follicle and per unit surface area, at Days 5-7 and 11-13 only. Intermediate "spring" follicles exposed to GTH had similar E_2 production per unit surface area as large follicles at Days 5-7 and 30-32; only at Days 11-13 was E_2 production per unit surface area by large "spring" follicles significantly less than by intermediate follicles. Hontela (1982) showed that the ovaries of mature goldfish (GSI= 12.5%-9.9%) after 30-32 days at 16L:8D / 20°C in March had about twice as many tertiary yolk globule stage (or "large-sized follicles") as primary or secondary stage oocytes, but the yolk vesicle stage oocytes were equally abundant as tertiary yolk globule oocytes; however, after 5-7 and 11-13 days of acclimation to 16L:8D / 20°C in March she found that tertiary yolk oocytes were as abundant as primary and secondary yolk globule oocytes, and that the tertiary yolk oocytes were twice as abundant as yolk vesicle stage oocytes. Intermediate follicles used in the present study consisted largely of yolk vesicle and primary yolk globule stage oocytes, and, since each of these stages were as abundant as tertiary yolk globule oocytes at certain times during the experimental period (Hontela, 1982), it is likely that the relative contribution by large and intermediate-sized follicles to T and E_2 in circulation in the spring could be due equally to each size range and category of these follicles. Hence, the maintenance and augmentation of elevated T and E_2 in female goldfish (Kagawa *et al.*, 1983b) at the end of April could be due to the recruitment of more large follicles from intermediate follicles as reflected by the high GSI at this time of the year (Yamazaki, 1965) and to the ability of large and intermediate-sized follicles to equally secrete T and E_2 in

response to GTH stimulation.

It is interesting to note that, comparing between "winter" and "spring" fish, T production per follicle and per unit surface area by GTH-treated large and intermediate follicles showed no variation in levels after 5-7 and 11-13 days of acclimation to 16L:8D / 20°C; at 30-32 days, large follicles from "winter" fish had greater production of T per follicle and per unit surface area than large follicles from "spring" fish. The output of E_2 per follicle and per unit surface area by large follicles under GTH stimulation declines from "winter" to "spring" when exposed to warm water and long photoperiod for different lengths of time; however, the E_2 levels produced per follicle and per unit surface area by intermediate follicles were generally similar between "winter" and "spring". Schreck and Hopwood (1974) have shown that seasonal levels of androgens in the female goldfish under natural conditions remain fairly constant at 10 ng / ml of plasma throughout the mid-winter and early spring months after reaching a peak of 33 ng / ml in early fall. Kagawa *et al.* (1983b) showed that plasma E_2 levels in goldfish gradually increase during mid-winter, corresponding with the period of increasing oocyte growth, and remain at very high levels during and after ovulation in April. A good seasonal correlation between *in vivo* plasma T and E_2 levels in amago salmon and *in vitro* production of these steroids by isolated ovarian follicles under GTH stimulation has also been reported (Kagawa *et al.*, 1983a). The fact that large follicles from goldfish exhibit a change in E_2 output would support the possibility that large "spring" follicles are preovulatory and might therefore show an ovulatory response to GTH in spring. As has been previously stated, estrogen secretion may be inhibited in these large follicles so that steroid production is prepared for production of progestogens appropriate for ovulation (Scott *et al.*, 1983; Kagawa *et al.*, 1983a, 1984). Therefore, the general lack of differences in E_2 production per follicle and per unit surface area by GTH-treated intermediate and small follicles exposed to 16L:8D / 20°C in "winter" and in "spring" could be attributed to their earlier stage of development, in contrast to the situation with the large follicles. Ovarian fragments taken from rainbow trout at the peak of exogenous vitellogenesis showed the longest amplification of E_2 output, when exposed to GTH pulses *in vitro*, compared to ovaries from fish in early recrudescence or in a mature state (Zohar *et al.*, 1982). Also, the capacity of the rainbow trout ovaries to synthesize estrogens from labelled precursors *in*

vitro increased from the period of endogenous vitellogenesis through to the period of exogenous vitellogenesis, and correlated well with increasing plasma titers of E_2, E_1(estrone) and vitellogenin (van Bohemen and Lambert, 1981). Intermediate and small follicles in the present study predominantly consist of oocytes at early and middle stages of yolk deposition; hence, the high requirement of these oocytes for hepatically- synthesized yolk proteins is maintained by a constant output of T and E_2 under GTH stimulation in "winter" and in "spring". It is most likely that the parallel increase in GSI and plasma T and E_2 levels in female goldfish observed from January to April by Kagawa *et al.* (1983b) could partially be attributed to this accelerated growth of intermediate follicles and to their lack of any seasonal variation of E_2 output. The relatively higher production of T and E_2 per unit surface area and per follicle by large "winter" and "spring" follicles in contrast to intermediate follicles could also serve to maintain the process of accelerated growth of intermediate follicles under these conditions. High levels of T produced by large follicles in "winter" and "spring" under GTH stimulation could also provide an additional source of substrate for further aromatization by intermediate and small follicles, especially during the spring-spawning months when the continuous recruitment of more large preovulatory follicles from smaller-sized follicles occurs.

Ovarian follicles from "winter" and "spring" fish maintained for different lengths of time at 12L:12D / 12°C were also incubated for 4 hours at 12°C with GTH to test their steroidogenic responsiveness. T production per mg of protein, per unit surface area and per follicle by GTH-exposed large "winter" and "spring" follicles were fairly similar at Days 6-8 and 30-32; in general, GTH significantly stimulated T production by large follicles at Days 30-32 only. While E_2 production per mg of protein, per unit surface area and per follicle by GTH-treated large "winter" follicles at Days 6-8 were greater than at Days 30-32, E_2 levels were generally low and similar to their untreated control groups, indicating a very low level of responsiveness to GTH stimulation of large "winter" follicles under cold conditions.

Although significantly greater than untreated controls, there was a similar and relatively low E_2 production per unit surface area and per follicle by GTH-exposed large "spring" follicles from fish held at 12L:12D / 12°C, suggesting that their responsiveness to GTH does not vary under these conditions. Only when E_2 levels were expressed on a per

mg of protein was the production by GTH-treated large "spring" follicles at Days 6-8 greater than at Days 30-32. It is interesting to note that while GTH was unable to stimulate E_2 production per mg of protein, per unit surface area and per follicle by large "winter" follicles over their untreated controls throughout the 32-day experimental period, GTH did stimulate E_2 output by large "spring" follicles in comparison to their untreated controls, indicating that these follicles in "spring" maintain their responsiveness to GTH even late during the season (June-July). The physiological advantage and significance of maintaining a higher than basal level of responsiveness to GTH stimulation among large "spring" follicles in June-July rather than in March when goldfish should encounter more favorable ovulatory and spawning conditions is not known.

The T production per mg of protein, per unit surface area and per follicle by GTH-treated intermediate "winter" and "spring" follicles were also low and similar at Days 6-8 and 30-32 of 12L:12D / 12°C; GTH caused a significant stimulation of T production over their untreated controls in these follicles at only Days 30-32. Similarly, E_2 production by GTH-treated intermediate "winter" and "spring" follicles were low and constant throughout the 32-day experimental period and, except when E_2 production was expressed on a per mg of protein basis, GTH-treated follicles at Days 30-32 had consistently greater E_2 output per unit surface area and per follicle than their untreated controls. These observations could therefore indicate that the responsiveness of intermediate "winter" and "spring" follicles to GTH also does not vary under these conditions. Again, there appears to be no reason to explain the generally higher E_2 production levels per unit surface area and per follicle by these follicles under GTH stimulation in "winter" and "spring" over their untreated controls at Days 30-32 although it can be an indication that a long-term exposure to 12L:12D / 12°C in March and June-July stimulates their responsiveness to GTH in anticipation of a possible shift to more favorable environmental conditions which are appropriate for rapid oocyte growth and development.

When exposed to GTH, large follicles from fish acclimated to 12L:12D / 12°C for 6-8 days in March produced more E_2 per unit area and per follicle than intermediate follicles, suggesting that large follicles are more responsive to GTH stimulation. Hontela (1982) showed that after goldfish (GSI= 8.5%-7.7%) were exposed for 6-8 and 30-32

days to 12L:12D / 12°C in March the ovaries consisted largely of primary and secondary yolk globule stage oocytes; yolk vesicle and tertiary yolk globule oocytes were equally abundant but were less frequent than primary and secondary yolk globule oocytes. Although large follicles (tertiary yolk globule oocytes) were more responsive than intermediate follicles (yolk vesicle and primary yolk globule oocytes) in terms of E_2 output per unit surface are and per follicle at Days 6-8, because of the relative number of the different oocytes in the ovary under these conditions, it is likely that a greater proportion of circulating E_2 and T in female goldfish in March is due to the contribution by intermediate follicles in the goldfish ovary. A similar situation is also likely after 30-32 days of exposure to 12L:12D / 12°C in March, because of the greater relative abundance and the greater surface area of intermediate follicles over large ones.

The ability of GTH to continuously stimulate steroid production in all follicle sizes could be readily observed throughout the 32-day Long-Term Experiments under 16L:8D / 20°C and 12L:12D / 12°C in "winter" and "spring". The continuous output of T and E_2 supports the data of Stacey *et al.*(1983) and Kagawa *et al.* (1983b) that T and E_2 remain elevated around the time of ovulation in goldfish. Hence, a surge of GTH leading to ovulation of large preovulatory follicles may also stimulate a steroidogenic response among the remaining non-preovulatory follicles present in fish displaying an asynchronous pattern of oocyte growth. In contrast, E_2 plasma levels in salmonids decline before final oocyte maturation at the same time that GTH levels are increasing (Fostier *et al.*, 1978; Scott *et al.*, 1983).

The continuous production of T and E_2 by all follicle sizes from "winter" and "spring" fish under GTH stimulation leads to the question of how rapid the metabolic clearance rate is of these steroids *in vivo* and what the possible physiological significance of these steroids is at the time of ovulation and spawning. The role of T and E_2 in the final stages of oocyte maturation is not clear. Whereas E_2 is entirely ineffective, only pharmacological doses of T are effective in inducing germinal vesicle breakdown (GVBD) *in vitro* (Goetz and Bergman, 1978) and it has been suggested that T may only enhance the rate of GVBD in response to pituitary GTH (Young *et al.*, 1982). Other steroids are known to increase the effectiveness of the maturation-inducing steroid (17 alpha,20 beta-P), most notably 11-deoxygenated and 11-oxygenated corticosteroids (Goetz, 1983). There

has been very little research done to investigate the metabolic clearance rate of steroids in fish. It is known that the plasma clearance rate of GTH is influenced by the ambient temperature and the stage of sexual maturity of the fish (Cook and Peter, 1980a).

C. Effect of Incubation Temperature on Steroid Production by Goldfish Ovarian Follicles *In Vitro*

The results of this experiment are presented in Figs. 22 and 23 and demonstrate that temperature has a direct effect on follicular steroidogenesis. Levels of E_2 and T produced by GTH-treated follicles incubated at 20°C for 4 hours were three to five fold greater than levels produced following a 4 hour incubation at 12°C. The levels of steroid produced at 20°C incubation over 12°C are greater than what can theoretically be predicted on a Q10 or a "temperature coefficient" basis since most biological systems would have a coefficient close to 2. Kime (1982) has shown that the production of testosterone glucuronide from tritiated testosterone by goldfish testes *in vitro* increased with incubation temperature and the rate of production of steroid metabolites over a 10°C range was closer to the theoretical Q10 of 2. It may be that steroidogenic enzymes in goldfish ovarian follicles are more sensitive to shifts in temperature than are testicular enzymes and that steroid production cannot be simply predicted on the basis of Q10=2. Although the 4 hour incubation *in vitro* at 12°C may not have been sufficient for the follicles to reach their maximum level of steroid production, it is clear that low temperature does slow ovarian steroidogenesis more than would have been predicted. Bogomolnaya and Yaron (1984) have recently suggested that the *in vitro* estrogen-synthesizing system of *Sarotherodon aureus* is temperature-dependent, supporting their observations that elevated temperatures induce ovarian recrudescence in this species under natural and experimental conditions. Also, the observation by Kagawa *et al.* (1983b) that plasma T and E_2 levels in the female goldfish kept under natural photoperiod and temperature conditions were low from December to January before rapidly increasing in March and April agrees well with the present *in vitro* results that the gradual increase in ambient water temperatures from winter to spring influences circulating levels of these steroids.

The present data where follicles incubated with GTH at 20°C had higher steroid output than follicles incubated at 12°C may in part be explained by an increased rate of GTH uptake by follicular receptors at the warmer temperature. Cook and Peter (1980b) demonstrated that the *in vivo* ovarian uptake of labelled GTH by sexually maturing goldfish at 20°C was greater than the uptake found in fish held at 12°C which supports their hypothesis that a rapid rate of gonadal recrudescence at warm temperatures results from a greater level of ovarian responsiveness to endogenous GTH at 20°C than at 12°C. Hence, it is possible that warm temperatures can induce a conformational structural change of membrane-bound ovarian GTH receptors, resulting in increased affinity to GTH, or that there is an increase in density of GTH receptors at the higher temperature, thereby influencing the dynamics of GTH binding.

D. General Discussion

The results of the present investigation described some influences of environmneta! factors on follicular steroidogenesis in a teleost species. In particular, the present study showed some effects of time of day, season, length of acclimation to a given environmental regime and incubation temperature on the production of T and E_2 by goldfish ovarian follicles stimulated by GTH *in vitro*.

The Short-Term Experiments performed in "winter" (January) and in "spring" (April) showed that large, intermediate and small follicles had significant differences in their steroidogenic responsiveness to GTH at two times during the day. In "winter", all follicle sizes were more responsive at 1100h than at 1900h. However, only large "spring" follicles exhibited a temporal responsiveness to GTH stimulation. These results provide additional evidence that teleost gonads vary in their daily responsiveness to GTH as detected *in vivo* (de Vlaming and Vodicnik, 1977; Peter *et al.*, 1982) and *in vitro* (Kuo and Watanabe, 1978).

The results of the Long-Term Experiments wherein goldfish were acclimated to 16L:8D / 20°C for different lengths of time in January-February ("winter") and in May-June ("spring") demonstrated that this environmental condition exerts a different effect on the various follicle sizes in the goldfish ovary depending on the length of acclimation, the stage of sexual maturity of the fish and on the time of the year when such conditions are

introduced. A short-term (5-7 days) and a long-term (30-32 days) exposure of goldfish to long photoperiod and warm water in winter promoted follicular steroidogenesis by large follicles under GTH stimulation; whereas, the steroidogenic responsiveness of large "spring" follicles to GTH treatment progressively declined with an increase in length of acclimation. These observations support the suggestion that the decline in the E_2 output by each large follicle from "winter" to "spring" is a reflection of the onset of preovulatory steroidogenesis as the fish encounters conditions suitable for ovulation and spawning or is an indication of a decreased level of responsiveness to GTH stimulation due to atresia. Intermediate "winter" follicles had constant levels of E_2 produced per unit surface area and per follicle throughout the 32-day experimental period. High levels of E_2 were attained by intermediate "spring" follicles after 11-13 days of acclimation; however, their E_2 output per unit surface area and per follicle was generally similar to levels attained in "winter", indicating that intermediate follicles maintain a constant level of responsiveness to GTH stimulation in order to maintain their development.

The present investigation also indicated that the relative contribution of the different follicle sizes to circulating steroids in the female goldfish varies during the seasonal reproductive cycle. Due to their abundance in the winter and to their greater surface area exposed to circulating GTH, a greater proportion of T and E_2 in circulation under these conditions is due to the contribution of follicles of smaller diameter rather than to the more responsive large follicles (tertiary yolk globule oocytes). However, large and intermediate follicles in the spring would likely contribute equal amounts of T and E_2 in circulation as the goldfish ovary largely consists of these two follicle sizes at this time of the year.

In the other series of Long-Term Experiments, large "winter" follicles incubated with GTH at 12°C for 4 hours had higher E_2 production levels per mg of protein, per unit surface area and per follicle at Days 6-8 than at 30-32 days of acclimation to 12L:12D / 12°C, although these levels were low and similar to their untreated control groups. Similarly, T and E_2 production by GTH-treated large "spring" follicles were low and constant throughout the duration of the 32-day experimental period; however, a significant stimulation by GTH was generally observed over their untreated controls during all sampling times. The T and E_2 output by intermediate follicles from "winter" and "spring"

fish were likewise low and constant throughout the 32-day experimental period but generally showed a greater level of responsiveness to GTH in contrast to their untreated controls. A large proportion of circulating T and E_2 in the female goldfish maintained at 12L:12D / 12°C in March and, possibly in June-July, is contributed by intermediate follicles during the course of the experimental period. Taken together, these results indicate that the level of steroidogenic responsiveness of follicles to GTH stimulation is low under 12L:12D / 12°C conditions in March and in June-July. Moreover, the results of the incubation temperature experiment support these findings by suggesting that low temperatures reduce the rate of follicular steroidogenesis *in vitro*.

In conclusion, the present investigation has shown that environmental conditions exert a direct effect on the ovarian follicles of goldfish by influencing their steroidogenic responsiveness to GTH stimulation. Other endogenous factors such as hormones of hypothalamic, pituitary, follicular or non-follicular origins could also influence the steroidogenic capacity of goldfish ovarian follicles; however, the interaction of these factors with a given set of environmental conditions in the regulation of ovarian steroidogenesis in teleosts remains to be investigated.

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